The infection biology of pig-associated *Salmonella*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by Georgina Crayford

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List of Abbreviations

ACSSuT	Ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracycline
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CBA	Cost-to-benefit analysis
CFU	Colony-forming units
DAPI	4',6-diamidino-2-phenylindole
DST	Diagnostic sensitivity test
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DT	Definitive phage type
EBU	Evans Blue uranine
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
GALT	Gut-associated lymphoid tissue
GB	Great Britain
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTPase	Guanosine triphosphatase
IEC	Intestinal epithelial cell
IFN	Interferon
IκB	Inhibitor of kappa B
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LB	Luria Bertani
LRR	Leucine-rich repeat

LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Derby canine kidney
MDR	Multi-drug resistant
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MLVA	Multilocus variable number tandem repeat analysis
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NCP	National Control Programme
NF-κB	Nuclear factor-kappa beta
NOS	Nitric oxide synthase
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
pBD	Porcine β-defensin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFGE	Pulsed-field gel electrophoresis
PI	Propidium iodide
PP	Peyer's patches
PRR	Pattern-recognition receptor

PT	Phage type
PVDF	Polyvinylidene fluoride
QMRA	Quantitative Microbial Risk Assessment
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
SCV	*Salmonella*-containing vacuole
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SGI	*Salmonella* genomic island
SNP	Single nucleotide polymorphism
SPI	*Salmonella* Pathogenicity Island
spv	*Salmonella* plasmid virulence
STM	Signature-tagged mutagenesis
T3SS	Type III secretion system
TEER	Trans-epithelial electrical resistance
TEM	Transmission electron microscopy
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TAE	Tris-acetate-EDTA
U	Undefined phage type
UK	United Kingdom
ZAP	Zoonoses Action Plan
ZNCP	Zoonoses National Control Programme

Abstract

Non-typhoidal serotypes of *Salmonella enterica* remain important foodborne pathogens worldwide and the frequent emergence of epidemic strains in food-producing animals is a risk to public health. In recent years, *Salmonella* 4,[5],12:i:- isolates expressing only the first phase of the two flagellar antigens (FliC) have emerged and increased in prevalence worldwide. In Europe, the majority of 4,[5],12:i:- isolates belong to phage types DT193 and DT120 of *Salmonella* Typhimurium and pigs have been identified as the reservoir species.

In this study, a number of pig-derived monophasic (4,[5],12:i:-) and biphasic DT193 isolates were characterised for phenotypes relating to virulence, to improve understanding of their ecological success. Additionally, their ability to invade a porcine intestinal epithelial cell line (IPEC-1) and stimulate a pro-inflammatory response from the host cells was investigated, to determine the infection biology of these strains. Monophasic and biphasic isolates were compared throughout, with the aim of identifying an explanation for the selective pressure behind the loss of flagellar phase variation.

It was found that the panel of DT193 isolates possessed a heterogeneous repertoire of virulence-related phenotypes and genotypes. A number of isolates demonstrated the ability to form biofilms, however the optimum temperature and time for expression of this phenotype varied among the isolates, which may have implications for bacterial survival in the environment and in the host. Another variation was in the presence of *sopE*, the gene for an important SPI-1 secreted effector protein associated with virulence, in the genomes of the isolates. The 4,[5],12:i:- isolates exhibited comparable adhesion and invasion to that of the virulent *S*. Typhimurium isolate 4/74, suggesting that these strains may be capable of colonising the small intestine of pigs *in vivo*. Infection with 4,[5],12:i:- and biphasic DT193 isolates resulted in approximately the same level of TLR-5 (a flagellin receptor) and IL-8 (a pro-inflammatory chemokine) mRNA upregulation, except in the case of one 4,5,12:i:- isolate that elicited significantly greater upregulation of these genes. The monophasic variants also elicited similar levels of caspase activation and cytotoxicity to the phase variable DT193 isolates.

These results suggest that monophasic *Salmonella* display a similar infection biology to phase variable *S*. Typhimurium during colonisation of the porcine intestinal tract. Consequently, failure of 4,[5],12:i:- isolates to express a second phase of flagellar antigen (FljB) is unlikely to hamper their pathogenicity during colonisation of the porcine intestinal tract *in vivo*.

1 Introduction

1.1 *Salmonella* species

1.1.1 Discovery

The genus *Salmonella* was named after Daniel E. Salmon who, working alongside Theobald Smith, discovered the bacterium subsequently named *Salmonella choleraesuis* in the intestine of a pig whilst searching for the causative agent of common hog cholera (Salmon & Smith, 1886). They proposed that the organism they had found was the agent they had been searching for, but later research eventually showed that hog cholera (also known as Classical Swine Fever) occurred as a result of a viral agent (De Schweinitz & Dorset, 1903). *Salmonella* was consequently neglected for several years until its potential to cause many different disease states in animals became apparent. Five years previous to Salmon and Smith's discovery, Karl Eberth, who is now recognised as the discoverer of the Typhi serotype, observed rod-shaped organisms in the spleens and lymph nodes of human typhoid patients (Marineli *et al.*, 2013).

1.1.2 Phylogeny

Salmonella are Gram-negative rod-shaped bacteria that are facultatively anaerobic. They belong to the Enterobacteriaceae family, in the Gamma-proteobacteria subdivision and the genus is recognised as having two distinct species: *bongori* and *enterica*. *Salmonella enterica* can be further divided into six subspecies (*enterica, salamae, arizonae, diarizonae, soutane* and *indica*), which are distinguishable by certain biochemical characteristics determined mainly by multilocus enzyme electrophoresis (MLEE; Beltran *et al*., 1988; Boyd *et al*., 1996). The evolutionary history of the *Salmonella* genus is summarised by Ellermeier and Slauch (2007). Briefly, the common ancestor of the bacteria acquired a type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) and the two species diverged when the *Salmonella enterica* ancestor acquired a second *Salmonella* pathogenicity island (SPI-2) T3SS. Further differentiation of the subspecies followed after. The nomenclature of *Salmonella* is complicated. Originally, serotypes of *Salmonella* were named after the disease profile they caused, for example *S.*

Typhimurium was named so because of its association with typhoid fever-like infection in mice and *S.* Choleraesuis because of its association with hog cholera. However, after the realisation that not all *Salmonella* had specific hosts, any new serotypes that were discovered were named according to the location at which they were found, *S*. Dublin for example. Over 2600 different serotypes of *Salmonella* have been identified. All serotypes mentioned in this thesis belong to *Salmonella enterica* subspecies *enterica*, hereafter referred to only as "*Salmonella*". For example, *Salmonella enterica* subspecies *enterica* serotype Typhimurium is described as *Salmonella* Typhimurium, or *S*. Typhimurium. The serotype (e.g. Typhimurium) is not italicised and starts with a capital letter to avoid confusion between serotypes and species (e.g. *enterica*).

1.1.3 Antigenic structure

Members of *Salmonella enterica* subspecies *enterica* are classified into serotypes, or serovars, according to their surface antigens, as determined by agglutination serology under the Kauffman-White scheme (Grimont & Weill, 2001). Newly isolated serotypes are now not usually given a name but are instead denoted by their antigenic formula. The antigenic structure of *Salmonella* consists of somatic (O), flagellar (H) and surface (Vi) antigens. O antigen is a component of the outermost lipopolysaccharide (LPS) layer of Gram-negative bacteria, oligosaccharide, which is a key interface for host-pathogen interaction. It is the most immunodominant constituent of the LPS and has been found to be highly variable among strains of *Salmonella*, with even subtle changes in structure producing notable effects on antibody recognition (Kim & Slauch, 1999). Because of this variability, the O antigen enables the Kauffmann-White scheme to divide *Salmonella* into 46 serotypes, depicted by numbers 1 to 67. H antigens are also highly immunogenic and can be found on the flagella that are distributed evenly across the surface of *Salmonella* cells. Again, because of the immunogenicity of H antigens there has been selection for variation in the flagellar subunits exposed on the surface. The flagellar filament is encoded by two different flagellin genes, which are expressed alternately through a mechanism known as "phase variation" (Andrewes, 1922). The first of the two flagellar phases is encoded by *fliC* and *fljB* encodes the second. Most *Salmonella* serotypes are biphasic and express both flagellin subunits, which have the same function but are immunologically different, whereas other strains are monophasic and only express one (usually phase 1) of the two

flagellin subunits. Some serotypes do not express H antigens at all and are consequently non-motile. Phase 1 flagellar (H) antigens are described, under the Kauffmann-White scheme, with lower case letters and phase 2 flagellar antigens by numbers. The final part of serotype classification under the Kauffmann-White scheme analysis describes the presence of the Vi capsule, a polysaccharide produced only by serotypes Typhi, Paratyphi C and sometimes Dublin. When reporting the serotype of *Salmonella* strains, the O antigen is written first, followed by the Vi antigen if present and then the H antigen(s), each separated by colons (Brenner *et al.*, 2000). For example, the antigenic structure of *S*. Typhimurium is 4,[5],12:i:1,2, where "4,[5],12" represent the O antigens present, "i" denotes the first phase H antigen and "1,2" denotes the second phase H antigen.

1.1.4 Phage types

Dominant serotypes, including *S*. Typhimurium and *S*. Enteritidis, can be further classified by phage typing, which evaluates the susceptibility of isolates to a panel of selected bacteriophages. Certain phage types are predominant in specific animal hosts, so phage typing of human isolates can help to determine the source of infection and thus aid epidemiological studies. The Colindale phage typing scheme for *S*. Typhimurium was first devised by Bessie Callow (Felix & Callow, 1943) and has since been developed to distinguish between more than 200 phage types using 37 bacteriophages (Anderson *et al.*, 1977). Under this scheme, some phage types cannot be fully validated as being stable and specific to serotype Typhimurium, so are referred to as undefined phage types (U) rather than definitive phage types (DT).

1.1.5 Host range

Salmonella serotypes can be classified according to their host range: host-adapted and host-restricted (Uzzau *et al.*, 2000). Host-restricted serotypes, for example *S.* Typhi in humans, typically cause a typhoid-like disease in a single host species. Host-adapted serotypes are typically associated with one host species but are capable of producing disease in other, usually related, host species. *S.* Choleraesuis and *S.* Typhisuis in pigs are examples of host-adapted serotypes. Finally, broad host range serotypes such as *S.* Typhimurium rarely cause systemic infections, but are able to colonise the gastrointestinal tract of a wide range of host animals. Depending on the host and serotype involved salmonellosis can result in

gastroenteritis, septicaemia and abortion. Serotypes of the *enterica* subspecies are those most commonly isolated in high numbers from animals and humans and they mostly cause acute self-limiting gastroenteritis. These serotypes are of most concern to public health because they are usually zoonotic.

1.2 *Salmonella* in humans

Salmonella is the second most frequently reported zoonosis in humans and is the foremost responsible agent of food-borne disease outbreaks in the European Union (EU; Anon., 2014a). In 2012 a total of 92,916 cases of salmonellosis in humans were confirmed in the EU, of which 8,812 were reported in the United Kingdom (Anon., 2014a). This was a reduced number compared to previous years, following a consistent decline in *S.* Enteritidis isolations (Figure 1.1), which is probably largely due to successful *Salmonella* control programs in poultry flocks, in particular laying hens. It is known that most cases of infectious intestinal disease, including salmonellosis, are not reported to national surveillance systems, so the infection rate is likely to be greatly underestimated (Tam *et al.*, 2012). The most common serotypes reported in humans in 2012 in the EU and Great Britain (GB) are detailed in Table 1.1.

Salmonella primarily reside in the digestive tract of infected hosts. The ubiquitous nature of *Salmonella* means that any food products can become contaminated and cause infection in humans, although usually it is those of animal origin that are responsible. Person-to-person transmission can also occur where the faeces of an infected person comes into contact with other humans and/or food items, usually through lack of hand-washing. This is because the bacterium is shed in the faeces of humans during both symptomatic and asymptomatic infection (Buchwald & Blaser, 1984). Following an incubation period of 12-72 hours, most people infected with *Salmonella* will experience self-limiting gastroenteritis with diarrhoea, vomiting and fever for up to 1 week. As with most other enteric infections young children, elderly people and the immunocompromised are at-risk groups and may experience more serious clinical outcomes. *S.* Typhi and a few other serotypes can disseminate further than the gastrointestinal tract in humans, leading to a systemic infection characterised by bacteraemia and septicaemia, known as typhoid fever. However, the majority of cases of salmonellosis in humans are a result of infection with non-typhoidal serotypes.

Table 1.1 Five most frequent serotypes of *Salmonella* confirmed in human cases in 2012.

EU (n = 82,409)*		GB (n = 8,653)†	
Serotype	**%**	**Serotype**	**%**
Enteritidis	41.3	Enteritidis	28.0
Typhimurium	22.1	Typhimurium	12.6
4,[5],12:i:-	7.2	4,[5],12:i:-	11.3
Infantis	2.5	Infantis	2.7
Stanley	1.4	Newport	2.4

References: *Anon. (2014a); †Anon. (2013a)

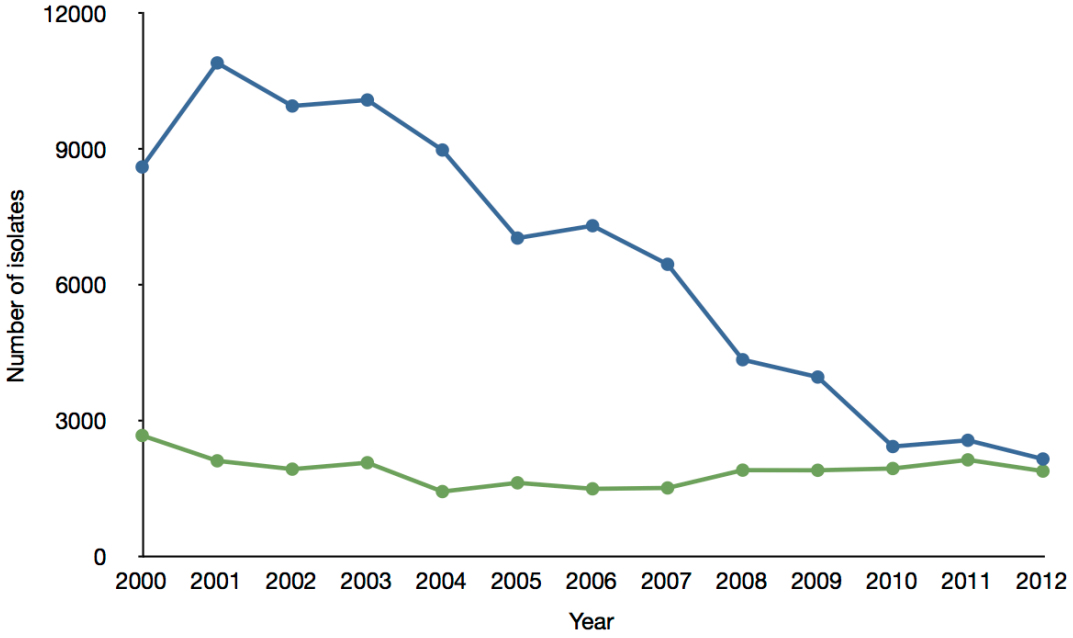


Figure 1.1 Human isolates of *Salmonella* reported to the Health Protection Agency Centre for Infections in England and Wales.

Number of human isolates of *S.* Enteritidis (●) and *S.* Typhimurium (●) reported in 2000-2012. Data source: Anon. (2013b).

1.3 *Salmonella* in pigs

1.3.1 Epidemiology

S. Choleraesuis infection in pigs usually causes septicaemia and symptoms include inappetance, lethargy and fever. Following an outbreak of *S*. Choleraesuis, pig producers can expect high mortality among the herd, but low morbidity (Reed *et al.*, 1986). *S*. Choleraesuis is now rarely reported in pigs in the UK, with only one incident reported in 2009 after a long absence (Anon., 2013a) but this was once not the case. In the 1950s and 1960s *S.* Choleraesuis accounted for up to 90% of all *Salmonella* isolates from pigs in the UK (Sojka *et al.*, 1977). Since then *S.* Typhimurium has replaced Choleraesuis as the dominant serotype in pigs across North America and Europe, including Britain (Figure 1.2). Infection with *S*. Typhimurium causes gastroenteritis in pigs. As with *S.* Choleraesuis infection, pigs infected with *S*. Typhimurium can be inappetant, febrile and lethargic, but they may also have watery, yellow diarrhoea, which is not apparent with septicaemia disease. Mortality is usually low and although morbidity can be high (Wilcock & Schwartz, 1992), more often the disease is subclinical or mild and transient (Davies, 2001).

During 2006 to 2007 an EU-wide baseline survey was carried out to determine the prevalence of *Salmonella*-infected pigs at the point of slaughter (Anon., 2008e). The average prevalence across all Member States was 10.3%, meaning that one in ten slaughter pigs were estimated to be infected with *Salmonella* in the ileo-caecal lymph nodes, where samples were taken from. However, there was wide variation between individual Member States, with prevalences ranging from 0.0% to 29.0%. The UK had an observed prevalence of 21.2%; double that of the EU average. This result correlates with that of a national survey carried out on slaughter pigs in Great Britain in 1999-2000, which estimated a *Salmonella* caecal carriage rate of 23.0% (Davies *et al.*, 2004b). A more recent prevalence survey conducted in 2013 in UK pigs at slaughter found *Salmonella* in 30.5% of caecal samples, suggesting that the level of *Salmonella* in commercial pigs could be rising in this country (Anon., 2014b). The five most frequently isolated serotypes in this baseline survey were, in decreasing order: 4,5,12:i:-, 4,12:i:-, Typhimurium, Derby and Bovismorbificans, all of which, with the exception of Bovismorbificans, are common causes of salmonellosis in humans. Carcass swabs collected at abattoir were also tested for *Salmonella* in this survey and 9.6% were found to be positive. Contaminated pig

meat has the potential to cause human disease, so it is important to control the prevalence of *Salmonella* in slaughter pigs.

In 2008 another baseline survey was conducted in the EU to estimate the prevalence of *Salmonella* in pig breeding holdings by sampling faeces (Anon., 2009). The overall EU prevalence of *Salmonella*-infected breeding pig holdings was estimated to be 31.8%, with variation among Member States ranging from 0.0% to 64.0%. The UK prevalence was again estimated to be much higher than the EU average, with 52.2% of breeding pig holdings shown to be positive. When data from across the EU was considered, *S*. Derby was the most frequently isolated serotype from breeding holdings, followed by *S*. Typhimurium, although in the UK this was reversed. A high prevalence of *Salmonella* in breeding pigs increases the potential for vertical transmission to the rest of the pig production industry through contamination of buildings and movement of live animals. The slaughter pig and breeding pig EU baseline surveys both utilised bacteriological culture of samples, which is a low sensitivity method (Davies *et al.*, 2000; Funk *et al.*, 2000), so the true prevalence is likely to have been underestimated by these studies.

Often several different serotypes and phage types of *Salmonella* can be found on a pig farm at any one time (Wales *et al.*, 2009), although the range usually narrows when testing at point of slaughter (Davies, 2001). Until recent years the most common serotypes isolated from British pigs were Typhimurium, Derby, London and Kedougou (Anon., 2008d) and the relative number of reports of these serotypes had remained largely unchanged since the mid-1990s (Gresham, 1996; Miller *et al.*, 2011). However, the incidence of monophasic variants of *S*. Typhimurium has increased in recent years, since they first appeared on British pig farms in 2005 (Anon., 2010a). This clonal group has now replaced *S.* Derby as the second most common serotype (Figure 1.2). Until 1992 the main phage types of *S*. Typhimurium isolated from pigs were DT193, DT208 and DT12 but a newly emerged multi-drug resistant strain, DT104, predominated during the 1990s (Gresham, 1996). The prevalence of DT104 has since declined in Great Britain, with U288 and DT193 now being the predominant phage types (Anon., 2013a; Miller *et al.*, 2011).

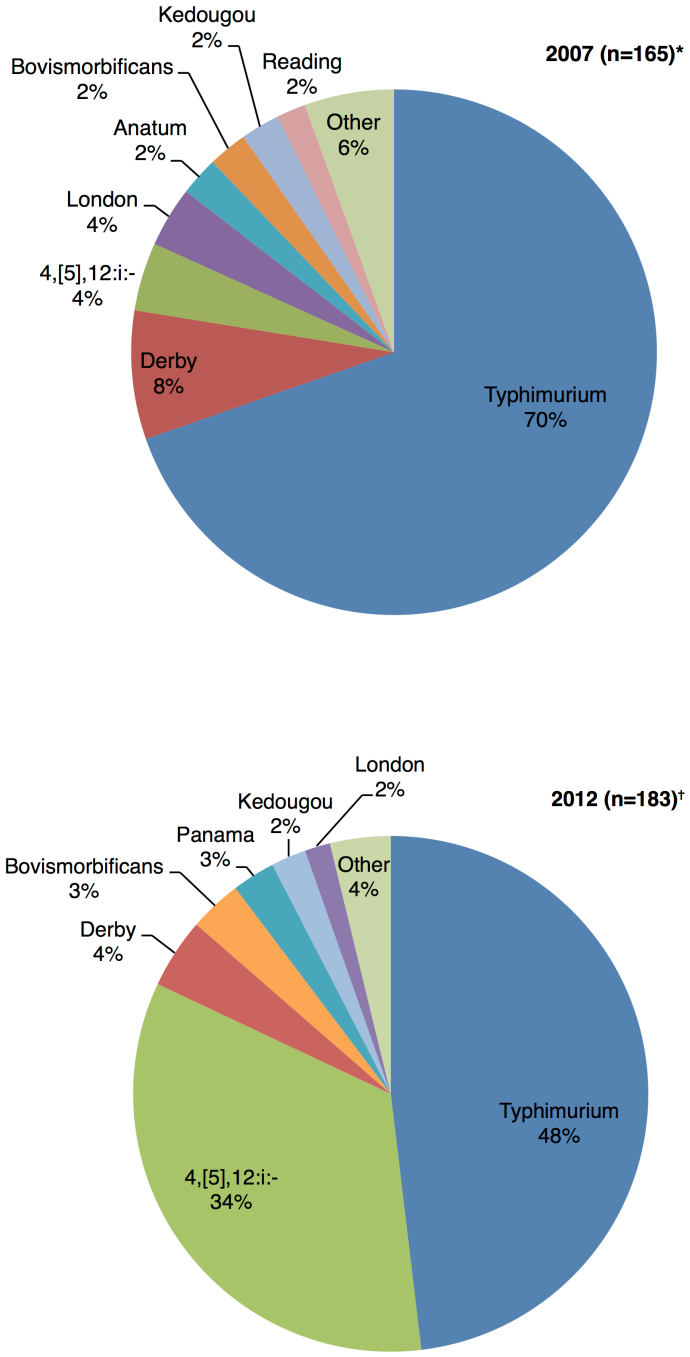


Figure 1.2 Isolations of the most common serotypes in pigs in GB.

The most common serotypes isolated from pigs in Great Britain in 2007 and 2012 as a proportion of the total number of isolations (n) in each year. Data source: *Anon. (2008d) †Anon. (2013a).

The widely accepted view is that transmission of *Salmonella* is by the faecal-oral route from pig-to-pig. However, other possible routes of transmission should also be considered including dust-borne, aerosol and nose-to-nose (Fedorka-Cray *et al.*, 1994; Fedorka-Cray *et al.*, 1995). Dissemination of the organism also requires that the bacteria survive outside of the host and *Salmonella* have been shown to persist for long periods in the environment within faeces (Rajtak *et al.*, 2012; Schwartz, 1999). For example, during their studies on pig farms, Baggesen *et al.* (1997) recovered *Salmonella* from faeces, pens, dust, equipment, ventilation structures and slurry. High levels of the organism have also been shown to persist in pig pens even after disinfection (Davies & Wray, 1997), demonstrating that *Salmonella* are hardy and ubiquitous bacteria. Rodents, insects, cats, birds, foxes, humans and contaminated feed have also been shown to play a role in the dissemination of *Salmonella* (Fedorka-Cray *et al.*, 2000).

Most experimental infection studies report that a large infective dose of 10^8 to 10^{11} *Salmonella* is required to produce moderate disease in pigs (*S.* Cholereraesuis: Gray *et al.*, 1996). However, it is expected that in field situations the initial infective dose is much lower, and Loynachan and Harris (2005) demonstrated this in their field study in which a dose of only 10^3 *S.* Typhimurium bacteria was able to induce acute infection. Pigs infected with *Salmonella* tend to shed the bacteria in low levels, usually intermittently, in their faeces (Kranker *et al.*, 2003; Nielsen *et al.*, 1995). Most adult pigs remain carriers, with low numbers of *Salmonella* persisting in the animal for at least 28 weeks post-infection without any symptoms being apparent (Wood & Rose, 1992). Reactivation of bacterial excretion can occur due to stress, for example during transport and lairage, or concurrent disease (Berends *et al.*, 1996). These subclinical carrier animals are believed to be the primary source of infection of other pigs and contamination of pig carcasses.

1.4 Public health significance

Although *Salmonella* infection in humans is mainly attributed to *S.* Enteritidis and *S.* Typhimurium, the majority of the other *S. enterica* serotypes are also capable of causing infection in humans. The ubiquitous nature of *Salmonella* and their ability to colonise the gut of food animals means that almost any food product, particularly those of animal origin, can act as a vehicle for human infection. Chance infection in livestock animals can result in contamination of a particular food product, which may

cause an outbreak of infection in humans. For example, an outbreak of *S*. Newport, usually an uncommon cause of human salmonellosis in the UK, was associated with consumption of cured ham (Lyytikäinen *et al.*, 2000). Conversely, persistent infection in certain food animal species can contribute to the underlying background levels of human *Salmonella* cases. In this respect, some animal reservoirs and food sources have a higher impact on human health than others.

A recent report estimated the relative contribution of different animal and food sources to human *Salmonella* infections in the EU (Pires *et al.*, 2011). Two methods were used to attribute human salmonellosis to the responsible food and animal sources, using data from the European Centre for Disease Prevention and Control of reported human *Salmonella* infections, along with serotype data collected as part of the EU-wide baseline surveys described previously and data reported by EU Member States of food-borne disease outbreaks. First, a microbial sub-typing model was applied to the data, resulting in attribution of human salmonellosis to four animal reservoirs: pigs, broiler chickens, laying hens and turkeys, with the relative contribution varying between regions and countries (Pires & Hald, 2010). This was based on the principle that certain *Salmonella* serotypes and phage types are strongly associated with specific animal reservoirs, due to clonal dissemination. The most important source in the EU was estimated to be the laying hen reservoir, followed by pigs, with 43.8% and 26.9% of human cases attributed to each source, respectively. The second source attribution approach involved analysis of data from outbreak investigations, which resulted in human salmonellosis being attributed to a total of 19 food sources and water (Pires *et al.*, 2010). Eggs were judged to be the most important source, followed by pork, chicken, general “meat and poultry” and dairy products. It must be borne in mind that this report was based on data collected from 2007 to 2009 and subsequently the contribution of eggs to human infection decreased. Therefore, it is possible that pork is becoming an increasingly important source of infection.

The two approaches used to estimate the contribution of different food and animal sources to human infection focused on different points along the farm-to-fork chain. The microbial sub-typing model attributed disease to the point of reservoir, whereas the outbreak analysis attributed disease to the point of exposure. The results of each were essentially in agreement, that chickens/eggs represent the foremost

important source followed by pigs/pork (Pires *et al.*, 2011). These estimates should be useful for the application of risk mitigation strategies. Presence of *Salmonella* on meat carcasses usually occurs as a result of faecal contamination during lairage, slaughter and processing. The level of carriage of *Salmonella* in the live animal and the hygiene of the processing plant will both determine the degree of carcass contamination. Until June 2014 *post-mortem* meat inspection of pig carcasses in the EU involved visual inspection, palpation and incision to detect and eliminate macroscopic abnormalities that could pose a threat to human health. However, the zoonotic diseases that can be detected by *post-mortem* inspection, such as tuberculosis, trichinosis and brucellosis, have become controlled in many areas due to modernisation of animal health care and husbandry. A report from the European Food Safety Authority (EFSA) identified and ranked the main hazards for public health that should be covered by meat inspection (Anon., 2011). A qualitative risk assessment of food-borne hazards was conducted using data on the prevalence of hazards on chilled carcasses, incidence and severity of disease in humans and attribution of hazards to pork as a source. *Salmonella* spp., *Yersinia entercolitica*, *Toxoplasma gondii* and *Tricinella* spp. were deemed to be the most relevant biological food-borne hazards to public health at present in the EU. It was concluded that the traditional *ante*- and *post-mortem* inspection procedures did not enable detection of these biological hazards and the palpation and incision techniques mediated bacterial cross-contamination. To reduce the risk to humans from these zoonoses a new EU regulation was introduced to amend the *post-mortem* inspection requirements for domestic swine to visual-only inspection.

1.5 Control measures

1.5.1 National control programmes

Under EU Regulation (EC) 2160/2003 all Member States are required to implement effective measures for the detection and control of *Salmonella* and other zoonotic agents throughout all relevant stages of production. The regulation requires that Member States identify specific targets for the reduction of the prevalence of specified zoonoses, including *Salmonella* spp., in specified animal populations. Specific National Control Programmes (NCPs) designed by individual Member States and approved by the European Parliament must then be employed to meet

the targets set for reduction of prevalence. Six 12-month surveys were completed in order to establish the baseline prevalence of *Salmonella* in breeder, layer and broiler flocks of domestic fowl (*Gallus gallus*), turkey flocks, breeding pigs and slaughter pigs (Anon., 2005; Anon., 2007a; Anon., 2007b; Anon., 2008a; Anon., 2008b; Anon., 2008e; Anon., 2009). Based on these estimates of prevalence, Community targets for the control of Salmonella were set by the European Commission. Subsequently, UK NCPs aimed at achieving these targets were implemented for breeders, layers, broilers and turkeys. These NCPs include control of *Salmonella* in feed production, primary animal production and processing and preparation of food. They detail sampling regimes for the detection of *Salmonella*, the responsibilities of competent authorities and food and feed business operators, control measures to protect public health and evaluation of progress.

The European Commission has yet to set the Community target for pigs and it is not known at which point in the production chain the target will be set. The target will take into account results from the baseline surveys of breeding and slaughter pigs, plus recommendations from a quantitative microbiological risk assessment (QMRA; Anon., 2010b) and a cost-to-benefit analysis (CBA; Anon., 2010c). The QMRA models *Salmonella* in pigs from farm to consumption with the aim of assessing the impact of reductions of *Salmonella* prevalence in slaughter pigs and the impact of specific control measures applied at various points along the production chain on the number of human cases of salmonellosis. The QMRA predicts that an elimination of *Salmonella*-infected breeder pig herds and *Salmonella*-contaminated feed would result in a reduction of *Salmonella* prevalence in slaughter pigs, as these are two major sources of infection. According to the QMRA, specific interventions in the slaughterhouse would be more likely to produce greater reductions in human salmonellosis cases attributable to consumption of pork, in the short term; however, implementation of multiple interventions both on farm and in the slaughterhouse would result in more effective reductions in human cases. The European pig sector CBA model was developed to assess the economic profitability of pre-harvest control measures for *Salmonella* in terms of reductions of human health costs and benefits associated with improved pig productivity. Four intervention scenarios were analysed, ranging from basic on-going surveillance only, to a selection of targeted on-farm measures depending on prevalence. The CBA showed that none of the scenarios would result in an economically profitable return, suggesting that there

would be no economic benefit to setting targets to reduce *Salmonella* in slaughter pigs. However, the benefit-cost ratio may be improved under certain circumstances so it is suggested in the report that further exploration is done to find possible successful scenarios. For example, results may have been more positive if post-harvest measures, such as anal bunging and carcass decontamination, had been analysed. It should also be considered that there was a lack of precise data and information to include in the model, therefore accurate assumptions could not be made.

1.5.2 ZNCP

Until a NCP for *Salmonella* in pigs is established, the Zoonoses National Control Programme (ZNCP) is in place for the British pig industry. The ZNCP was introduced in April 2008 to replace the Zoonosis Action Plan (ZAP) *Salmonella* monitoring programme, which was started in 2002. Under the ZAP scheme, meat juice samples collected from slaughter pigs were serologically tested by an indirect lipopolysaccharide (LPS) enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to *Salmonella*, to estimate the burden of *Salmonella* on finishing farms (Armstrong, 2003). A positive test result indicated previous infection, while it was understood that pigs testing positive by serology were not necessarily infected and actively excreting *Salmonella* in their faeces at the time of slaughter. Pig farms were categorised into one of three groups based on their sample results and producers in Level 2 or Level 3, with medium and high estimated prevalence respectively, were required to develop an action plan for the control of *Salmonella*. The theory behind the ZAP scheme was that risk to public health would be reduced if carriage of *Salmonella* by slaughter pigs were limited. A target of 25% reduction within 3 years was set for the scheme. ZAP was based on a similar *Salmonella* surveillance and control programme operated in Denmark which, since it had begun in 1995, contributed to a decrease in the level of *Salmonella* in Danish pork and a consequent reduction in the number of human cases attributable to pork (Alban *et al.*, 2012; Nielsen *et al.*, 2001).

Despite a reduction in the number of positive samples by approximately 12% within the first 3 years of the ZAP scheme, this reduction was not sustained and there was an increase in the proportion of positive samples from 2006 to 2008 in England and Northern Ireland (Anon., 2008c). It was at this point that the ZAP scheme was

modified and improved to form the ZNCP. The new control programme aimed to reduce the risk of *Salmonella* to consumers of British pig meat by taking action at every level along the farm-to-fork chain, relying on feed companies, producers, hauliers and processors working together. The categorisation of pig herds into ZAP levels was abolished and now all producers with finishing units were expected to have detailed *Salmonella* control action plans. In addition to the serological testing of juices from meat samples, bacteriological testing of carcass swabs collected in abattoirs was done to monitor risk to consumers. However, despite these changes ZNCP failed to produce any effect on the prevalence of *Salmonella* antibodies in slaughter pigs (Anon., 2012b). The proportion of positive meat juice ELISA results remained essentially unchanged from 2008 to 2012, when serological testing was finally ceased. In July 2012 BPEX, who run ZNCP, launched an on-farm *Salmonella* risk assessment tool to help farmers identify the most effective control measures for their individual farm circumstances. It is presumed that use of this risk-assessment tool will remain the principal approach to reducing *Salmonella* prevalence in British pig herds until a NCP is approved and introduced.

1.6 Epidemic and multi-drug resistant *Salmonella*

1.6.1 DT104

The evolution of *Salmonella* has been punctuated by the emergence of epidemic and multi-drug resistant (MDR) variants. Two of the most notable examples are the international spread of *S*. Enteritidis, associated with chicken eggs and egg products and the emergence of MDR *S*. Typhimurium DT104 (Rabsch *et al.*, 2001; Rodrigue *et al.*, 1990). DT104 was first isolated in 1984 in the UK and spread rapidly during the 1990s to become the second most common *Salmonella* serotype isolated from humans and the most common from cattle (Davies, 2001). From the UK cattle population DT104 was transmitted to other livestock animals, including poultry, sheep and pigs (Humphrey, 2001). Human infection was associated with the consumption of chicken, pork sausages, beef and paté (Wall *et al.*, 1994). It also spread throughout Europe, North America and other countries across the world (Helms *et al.*, 2005; Threlfall, 2000). A widely accepted view was that DT104 isolates disseminated as clones from a single strain (Davies, 2001; Humphrey, 2001) with high levels of transmission from animals to humans. However, more

recent work using whole genome sequencing and phylogenetic analysis of a large collection of DT104 isolates from Scotland identified that, at least in that local area, the bacterium was largely maintained within animal and human populations separately, with only limited transmission between the two (Mather *et al.*, 2013).

Since reaching peak incidence in 1996 in the UK, there has been a continual decline in reported cases of DT104 in humans and livestock. This epidemic strain was a public health concern because of its ability to spread rapidly internationally and its antimicrobial drug resistance. The majority of DT104 isolates are pentaresistant, carrying the antibiotic resistance pattern ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline), the genes for which are chromosomally encoded (Threlfall *et al.*, 1994). However they also have the ability to acquire additional resistance genes. By 1998, 13% of DT104 isolates in the UK were also resistant to trimethoprim, due to acquisition of a plasmid and 16% displayed resistance to ciprofloxacin, due to chromosomal mutation (Threlfall, 2000). Similar resistance patterns were also identified in isolates of DT104 in the United States and Denmark (Glynn *et al.*, 1998; Mølbak *et al.*, 1999).

1.6.2 Multi-drug resistance

Antimicrobial resistance has also been identified in other phage types of *S.* Typhimurium. Prior to the DT104 global outbreak DT204c, which was epidemic in cattle and pigs, was found to be resistant to a number of antimicrobial drugs (Wray *et al.*, 1986). Similarly, *S.* Typhimurium DT193, which was responsible for major outbreaks in humans in the UK and Italy as a result of consumption of contaminated pork products (Maguire *et al.*, 1993; Pontello *et al.*, 1998), is also an MDR strain. This phage type was derived from a previously prominent phage type, CSSuT-resistant (chloramphenicol, streptomycin, sulphonamides, tetracycline) DT204, following acquisition of a plasmid encoding additional resistance to ampicillin and kanamycin (Threlfall *et al.*, 1980). DT193 was the most common phage type isolated from pigs and the second most frequently isolated phage type of *Salmonella* in humans in 2012 in Britain (Anon., 2013a). MDR *S.* Typhimurium isolates are common on commercial pig farms as evidenced by a study, which found that 67% of isolates from highly positive pig farms in England and Wales showed resistance to between four and nine antimicrobial drugs (Miller *et al.*, 2011).

1.6.3 *Salmonella* genomic island 1

The antibiotic resistance genes harboured by *S.* Typhimurium DT104 are located on a 43 kilobyte (kb) genomic island known as *Salmonella* genomic island 1 (SGI1). The complete nucleotide sequence of this region was elucidated by Boyd *et al.* (2001), showing that it contains 44 open reading frames (ORFs), many of which are homologous to other known genes. The chromosomal location of these antibiotic resistance genes is a concern because it may mean that they are stably maintained, even in the absence of selective pressure (Mulvey *et al.*, 2006). Another concern is that some of the resistance genes located in SGI1 reside in class 1 integrons, which are easily transferred between bacteria (Hall & Collis, 1995). Integrons function as gene expression cassettes because they are able to acquire various genes and supply the promoter for their expression. Different types of integrons exist, but resistance integrons carry cassettes of antibiotic resistance genes; horizontal transfer of these integrons can facilitate spread of antimicrobial resistance. SGI1 has been found in a number of other *Salmonella* serotypes (Amar *et al.*, 2008) and some believe the presence of this island confers increased virulence to the carrier isolate (reviewed by Mulvey *et al.*, 2006).

1.6.4 Plasmid-associated resistance

Some serotypes of *Salmonella*, including Typhimurium, Enteritidis, Choleraesuis, Gallinarum and Pullorum, contain serotype-specific virulence plasmids in addition to virulence factors located on the chromosome (Gulig, 1990). An example of a serotype-specific virulence plasmid is pSLT, which belongs to *S.* Typhimurium. Various isolates of *S.* Typhimurium of differing phage types and containing several plasmid variants, have been shown to carry a virulence-resistance hybrid plasmid, pUO-StVR2, which originated from pSLT (Guerra *et al.*, 2001; Guerra *et al.*, 2002; Herrero *et al.*, 2006; Herrero *et al.*, 2008; Hradecka *et al.*, 2008; Montero *et al.*, 2012; Rodicio *et al.*, 2011; Rodríguez *et al.*, 2008). This hybrid plasmid resulted from pSLT acquiring a complex DNA segment encoding a central resistance island, conferring resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline on various mobile genetic elements including a class 1 integron and several transposons (Herrero *et al.*, 2008). Isolates of *S.* Typhimurium carrying this hybrid plasmid have been detected across Europe in humans, livestock and food of animal origin, including pork and chicken (Montero *et al.*, 2012).

pUO-StVR2 is an example of an antimicrobial resistant derivative of a serotype-specific plasmid (Rodicio *et al.*, 2011). Another type of hybrid plasmid is a resistance plasmid containing virulence genes of serotype-specific plasmids. The *Salmonella* plasmid virulence (*spv*) locus, associated with severe systemic infection in humans and other animals due to its role in extra-intestinal intracellular proliferation (Barrow *et al.*, 1987; Chikami *et al.*, 1985; Danbara *et al.*, 1992; Gulig & Curtiss III, 1987; Heffernan *et al.*, 1987; Libby *et al.*, 1997; Wallis *et al.*, 1995), is present on all serotype-specific virulence plasmids. There are a number of examples of resistance plasmids that have acquired the *spv* locus and/or other genes carried by the serotype-specific virulence plasmids (Rodicio *et al.*, 2011). One example is the group of related plasmids found in the Spanish clone of monophasic (4,[5],12:i:-) variants of *Salmonella* that has emerged in recent years. These monophasic isolates display a number of different MDR patterns, with the resistance genes encoded on mobile genetic elements grouped together with *spv* genes, from the aforementioned virulence plasmid pLST, on large non-conjugative plasmids (García *et al.*, 2011; García *et al.*, 2014; Guerra *et al.*, 2001). There is concern over hybrid plasmids that carry *spv* genes together with resistance genes, because the *spv* locus confers virulence and the MDR confers resistance to the antibiotics necessary to treat the infection. Therefore, selective pressure for antimicrobial resistance can simultaneously select for virulence properties (Rodicio *et al.*, 2011). Even more worrying is the presence of these hybrid plasmids in monophasic variants, which display huge potential for widespread dissemination. As such, monophasic variants of *Salmonella* with hybrid plasmids express virulence, resistance and epidemic potential.

1.6.5 Dissemination of MDR strains

The veterinary use of antimicrobials (therapeutic and prophylactic) may serve as a selective pressure for the maintenance of MDR *S.* Typhimurium strains in food animals (reviewed by Rabsch et al., 2001 and Mathew *et al.*, 2007), however other factors may also contribute. For example, *S*. Typhimurium has a higher prevalence of antibiotic resistance strains compared to other serotypes, in which MDR is usually rare, suggesting that *S*. Typhimurium is more receptive to transferable DNA (Humphrey, 2001). Antimicrobial resistance can be gained through horizontal gene transfer by a number of different mechanisms including transformation, conjugation,

transduction and exchange of transposons. This genetic transfer of resistance genes between pathogenic and even commensal organisms can occur in the gut of animals (Johnson *et al.*, 2010; Paulsen *et al.*, 2003). Horizontal transfer of genetic elements encoding antimicrobial resistance genes is a public health concern because the genes could be passed onto bacteria, both commensal and pathogenic, of the human digestive tract. The presence of resistance genes in clusters or integrons on mobile genetic elements aids the spread of particular combinations, as evidenced by certain resistance patterns being associated with clonal groups of specific serotypes and phage types. Because of these gene clusters, selection for one resistance gene can inadvertently result in selection for resistance to other unrelated drugs (Mathew *et al.*, 2007).

These factors explain how prevalence of antibiotic resistance increases, but there is also the question of how clonal groups of resistant phage types are disseminated. Davis *et al*. (2002) proposed that the rapid worldwide spread of resistant strains may play a more significant role in maintenance of such strains than regional antimicrobial selection pressure. The same authors suggested that human travel is an important vehicle for global dissemination, more so than international movement of livestock. Multi-resistant clones such as *S.* Typhimurium DT204, DT204c and DT104, and *S.* Enteritidis have typically declined in prevalence following their global dissemination, however the reason for this is not currently known.

1.7 Monophasic *Salmonella*

1.7.1 Classification

Monophasic variants of *Salmonella* have been isolated with increasing frequency from animals and humans worldwide since the 1990s. These strains cannot be fully typed by conventional serotyping, however serological characterisation demonstrates they have near-identical serotypes to *S.* Typhimurium. For example, the antigenic formula of *S.* Typhimurium is 4,[5],12:i:1,2 where “4,[5],12” indicates which O antigens it has, “i” describes presence of the first phase of H antigen, and “1,2” denotes presence of the second phase of H antigen. The square parentheses indicate epitopes that are variably present within a serotype, for example due to lysogenic conversion by bacteriophages. The most common monophasic variant is

4,[5],12:i:-. According to its antigenic notation, *Salmonella* 4,[5],12:i:- carries the same O antigens and first phase H antigen as *S*. Typhimurium, but lacks the second phase flagellar (H) antigen. Various molecular techniques used in a number of studies have identified common genetic factors in *S*. Typhimurium and monophasic strains, thus confirming that most isolates of this type of monophasic *Salmonella* are variants of *S*. Typhimurium (Achtman *et al.*, 2012; Alcaine *et al.*, 2006; Bugarel *et al.*, 2012; Echeita *et al.*, 2001; Guerra *et al.*, 2000; Laorden *et al.*, 2010; Soyer *et al.*, 2009; Zamperini *et al.*, 2007). However, it is likely that different isolates of 4,[5],12:i:- evolved through multiple independent emergence events, resulting in separate clonal groups with the same monophasic serotype but geographically distinct distributions (Soyer *et al.*, 2009). Monophasic isolates can also be phage typed and most identify as a recognised *S*. Typhimurium phage type. In the UK, recently emerged monophasic strains identified as DT193 are currently spreading rapidly, in a similar manner to the previous epidemic strain *S*. Typhimurium DT104 (Davies & Larkin, 2009).

1.7.2 Distribution

Other countries that are reporting a rapid emergence of monophasic strains include Spain, where the majority of isolates are classified as phage type U302 (De la Torre *et al.*, 2003; Echeita *et al.*, 1999), Thailand (Amavisit *et al.*, 2005; Boonmar *et al.*, 1998), Brazil (Tavechio *et al.*, 2004), United States (Agasan *et al.*, 2002; Alcaine *et al.*, 2006; Zamperini *et al.*, 2007), Canada (Mulvey *et al.*, 2013), Luxembourg (Mossong *et al.*, 2007), France (Bone *et al.*, 2010; Gossner *et al.*, 2012), Germany (Trüpschuch *et al.*, 2010), Belgium (Boland *et al.*, 2014) and Italy (Barone *et al.*, 2008). In the UK and other European countries the majority of outbreaks of monophasic serotypes in humans have been traced back to pork and pork products, and the strains are being isolated from pigs with increasing frequency, suggesting that pigs are the main species involved in the evolution of these strains and represent the main reservoir (Hopkins *et al.*, 2010). However, a significant number have also been isolated from cattle in the UK, along with occasional cases in sheep, horses, dogs and zoo animals (Davies & Larkin, 2009). In the United States a number of outbreaks in humans have been traced back to raw chicken and chicken products suggesting that poultry is involved in the emergence of these strains there (Zamperini *et al.*, 2007). The mechanism behind the rapid spread of these strains is unclear, however trade in pigs and pig meat could be a possible reason in Europe.

A previous epidemic strain, *S*. Typhimurium DT104, was able to spread rapidly due to its ability to cause host animals to shed large numbers of the organism in faeces more persistently than other strains (Evans & Davies, 1996), therefore enabling extensive environmental contamination and onward spread. It is possible these emergent monophasic strains have spread in a similar way.

1.7.3 Flagellar phase variation

As previously mentioned, isolates of monophasic *Salmonella* are phenotypically characterised by a lack of one of the phases of flagella expression. To understand the significance of this it is first necessary to describe the genetic basis of flagella in classical *S*. Typhimurium. The flagella on *S.* Typhimurium cells are composed of three distinct substructures: the basal body and transmembrane motor; a hook that links the motor and the filament; and the filament, which acts as a propeller. The filament is composed of thousands of subunits of flagellin protein, which are secreted by a flagellum-specific T3SS at the base of the flagellum (Macnab, 1999) and moved through the lengthening structure to be assembled on the distal tip. Two different flagellar filament proteins, FljB and FliC, can be alternately expressed by *S.* Typhimurium by phase variation, a process that was first described by Andrewes (1922). Flagellar phase variation is achieved by a site-specific DNA inversion event in the chromosome, which controls expression of certain flagellar genes (Zieg *et al.*, 1977; Zieg *et al.*, 1978). Transcription of the *fljB* gene, which encodes FljB flagellar filament protein, is directed by a promoter that also directs transcription of the *fljA* gene, which encodes a repressor of *fliC*. This *fljBA* promoter is flanked by the recombination sites *hixL* and *hixR.* A reversible recombination reaction between these two *hix* sites is catalysed by the site-specific recombinase Hin, along with the regulatory protein Fis (factor for inversion stimulation), resulting in a switch in the orientation of the promoter (Haykinson *et al.*, 1996). When the *fljBA* promoter is in the "on" orientation, *fljB* and *fljA* are cotranscribed, and consequently *fliC* is repressed by *fljA*, while FljB flagellin is produced. When the promoter is in the "off" orientation, neither fljB nor *fljA* are transcribed, allowing *fliC* to be expressed and FliC flagellin to be produced. In the past it was assumed that the inhibitor FljA repressed transcription of the *fliC* gene, however it is now known that FljA also acts post-transcriptionally (Bonifield & Hughes, 2003; Yamamoto & Kutsukake, 2006). FljA interacts with *fliC* mRNA to interfere with both transcription and translation of the adjacent coding sequence as well as assembly of the FliC protein into a

functional flagellum (Aldridge *et al*., 2006). It is possible that this regulation at the translational level is more favourable than transcriptional regulation alone because it allows inhibition of FliC expression from existing transcripts, after the fljBA promoter switches to the "on" orientation, and consequently a more rapid transition to filaments composed of the alternative FljB flagellin (Bonifield & Hughes, 2003).

Flagellin is a potent antigen that stimulates the innate immune response in host organisms and although the FljB and FliC flagellin proteins are almost entirely genetically identical, amino acids exposed on the surface are divergent, resulting in different antigenicities (Joys, 1985; Wei and Joys, 1985). The biological significance of flagellar phase variation in *S.* Typhimurium has not yet been determined, however Aldridge *et al*. (2006) propose that it enables bacterial cells to escape environments that are unfavourable to a particular flagellar type, for example when host animals have developed immunity to one type of flagellin. The same authors also suggested that the mechanisms involved in flagellar phase variation ensure that only one of the two flagellin proteins are expressed at a given flagellum, so that flagella composed of a mix of FljB and FliC are never produced. Escape from hostile environments would be impossible if both flagella types were expressed simultaneously. This coincides with the view that flagellar phase variation influences the virulence of *Salmonella,* including its growth and survival within the host (Ciacci-Woolwine *et al.*, 1998; Ikeda *et al*., 2001; Yamamoto & Kutsukake, 2006).

1.7.4 Monophasic flagellar expression

Genetic characterisation of a number of monophasic isolates has revealed how the genetic basis of their flagella is different to that described above. For example, use of a microarray to characterise four monophasic isolates (4,[5],12:i:-) of Spanish origin identified a large chromosomal deletion of 16 genes including the entire *fljBA* operon: *fljB, fljA and hin* (Garaizar *et al*., 2002). Isolates of 4,[5],12:i:- from dairy cattle and poultry in the United States were characterised by polymerase chain reaction (PCR) and colony blot techniques, which revealed partial deletions of *fljB* in some of the isolates and complete deletions of *fljB* in others, with different isolates exhibiting different deletion patterns (Zamperini *et al*., 2007). Some of the isolates in this study were even identified as being positive for *fljB* suggesting that the mutation event responsible for the phenotype of these monophasic isolates is more subtle, possibly a point mutation or small deletion. An alternative explanation is that *fljBA* is

"locked" in the "off" orientation resulting in expression of *fliC* only and permanent repression of *fljB* transcription despite the gene being present. Similarly, Soyer *et al*. (2009) identified four monophasic isolates from the United States that were shown by PCR to contain intact *fljA, fljB* and *hin* genes. All of these isolates were identified as having an identical *fljB* sequence containing just one synonymous single-nucleotide polymorphism compared to the *fljB* sequence of *S.* Typhimurium (LT2 strain), which coincides with the suggestion by Zamperini *et al*. (2007) that simple mutations in *fljB*, not just complete deletions, may also cause a 4,[5],12:i:- phenotype. Bugarel *et al*. (2012) reached a similar conclusion after multiplex real-time PCR revealed presence of *fliC* and *fljB* in a number of 4,[5],12:-:1,2 (lacking phase 1 flagellin) and non-motile isolates, suggesting expression of these genes is blocked by mutation or deletion in the *fljBA* operon.

Analysis of the genome sequence of a 4,5,12:i:- animal isolate from the United States revealed a deletion of a larger fragment than that of the Spanish monophasic isolates investigated by Garaizar *et al*. (2002), including *fljB* and *fljA*, although unlike the Spanish isolates this variant retained the *hin* gene (Soyer *et al*., 2009). This further demonstrates that different monophasic isolates can belong to one of a number of different clonal groups with distinct genome deletion/mutation patterns. The emergence of monophasic variants could therefore be due to multiple independent deletion events affecting different *S.* Typhimurium ancestors in a variety of locations. An alternative theory is that the evolution of 4,[5],12:i:- isolates may represent a single emergence event, followed by subsequent mutations and/or deletions in the region of the genome surrounding the *fljBA* operon, thus giving rise to different monophasic lineages (Moreno Switt *et al*., 2009). Regardless of which theory is correct, it seems there is strong selective pressure for loss of phase 2 flagella but the reason for this is currently undetermined.

1.7.5 Ecological success of monophasic variants

It is somewhat surprising that monophasic variants are ecologically successful. As previously mentioned, phase variation between two flagellin types is thought to be an important feature of *Salmonella* pathogenesis, because having a subpopulation, with different flagellin antigens, should enable the bacteria to partially evade the immune response of the host (Aldridge *et al*., 2006). However, the ecological success of monophasic isolates may be due to complete silencing of expression of

flagellar antigens that are be recognised by the host immune system, thus giving them a selective advantage (Moreno Switt *et al*., 2009). Another selective advantage may be provided by the MDR profiles exhibited by the majority of monophasic isolates from Europe (Echeita *et al*., 1999; Garcia-Feliz *et al*., 2008; Lucarelli *et al*., 2010; Mossong *et al*., 2007). However, most of the monophasic isolates that have spread in non-European countries such as the United States and Brazil appear to be pan-susceptible or resistant to only a few antimicrobials (Agasan *et al*., 2002; Tavechio *et al*., 2004), suggesting that MDR can only partially account for the success of monophasic *Salmonella.* For example, gene deletions other than those involving the *fljBA* operon, as identified by Garaizar *et al*. (2002), may also contribute to a virulence-associated phenotype and aid rapid spread of monophasic strains.

1.8 *Salmonella* pathogenesis and virulence in pigs

1.8.1 Colonisation

A number of host defence mechanisms have evolved in the pig to reduce the likelihood of ingested *Salmonella* causing disease. Shi et al. (1999) detected porcine beta-defensin in the epithelium of the tongue, the concentration of which had antibacterial activity against *S.* Typhimurium. However, the palatine tonsils of pigs are often heavily infected in slaughter pigs (Wood *et al*., 1989) suggesting that bacteria overcoming this antimicrobial peptide are capable of colonising and persisting in the tonsillar crypts (Horter *et al*., 2003). The mechanism by which *Salmonella* colonises the tonsils and the virulence factors involved are not known and could be very different to the mechanism of invasion employed in the intestinal tract (Boyen *et al*., 2008a). One explanation for the high colonisation rate of the tonsils with *Salmonella* is the presence of microfold (M) cells in the epithelial lining of the follicular tonsillar regions, which enable uptake of the pathogen. This argument is supported by the evidence that a non-invasive strain was capable of colonising the tonsils (Boyen *et al*., 2006c)

Another host defence mechanism targets the bacteria that do not colonise the tongue but successfully pass to the stomach of the pig. In acidic conditions down to pH 3 *Salmonella* have been shown to produce acid shock proteins to survive (Foster, 1991), however certain areas of the porcine stomach can be as low as pH

2, so many bacteria are killed at this point. Any bacteria that survive pass into the small intestine. Nonspecific host protective mechanisms such as peristalsis and sloughing of intestinal epithelial cells (IECs) limit the colonisation of the intestine with *Salmonella* (Boyen *et al*., 2008a). Also, the presence of normal gut flora has been shown to inhibit intestinal colonisation by *Salmonella* and other enteropathogens, by Nurmi's concept of competitive exclusion. The types of mechanism by which protective flora control *Salmonella* infection include competition for limiting nutrients, competition for attachment sites on the mucosa and production of bacteriostatic short-chain volatile fatty acids (Nisbet, 2002; Nurmi *et al*., 1992). High concentrations of bile salts in the upper part of the small intestine are thought to inhibit invasion of *Salmonella* into IECs by repressing invasion gene transcription, resulting in marked reduction of proteins being secreted by the SPI-1 T3SS and consequently reduced overall invasive ability (Prouty & Gunn, 2000). The distal parts of the intestine including the ileum, caecum and colon, where bile salt concentration is much lower, are the main sites of *Salmonella* colonisation. Multiple bacterial factors are involved in *Salmonella* pathogenesis at these sites, including flagella, type 1 fimbriae and the aforementioned T3SS.

1.8.1.1 Flagella

Flagella are long helical filaments attached to rotary motors embedded within the membrane of the bacterial cell and have been shown to be involved in *Salmonella* colonisation by enabling the bacteria to translocate to sites of invasion (Misselwitz *et al*., 2012; Schmitt *et al*., 2001). Stecher *et al*. (2008) ascertained that flagella enhance *S.* Typhimurium fitness, but only in the inflamed gut, as demonstrated in a murine model of enterocolitis. They found that inflammation of the gut led to increased availability of high-energy nutrients in localised regions of the gut and flagella-based motility enabled the bacteria to move towards the source of nutrients, which resulted in faster replication.

1.8.1.2 Fimbriae

Once in the vicinity of the intestinal mucosa, attachment to host cells is an important first step during colonisation of the gut. Misselwitz *et al*. (2012) elegantly demonstrated that *S.* Typhimurium engages in "near surface swimming" along host cells to identify suitable target sites for invasion. This is followed by reversible binding mediated type 1 fimbriae (Lara-Tejero & Galán, 2009; Misselwitz *et al*.,

2011b). Fimbriae are hair-like structures found on the surface of *Salmonella* that carry virulence factors known as adhesins, which bind to mannosylated glycoproteins on eukaryotic cell surfaces and have been demonstrated to mediate attachment to various epithelial cell lines *in vitro* (Bäumler *et al.*, 1996d). Large numbers of fimbrial operons are encoded in the genomes of *Salmonella* serotypes, including *fim* (type 1 fimbriae), *saf* (*Salmonella* fimbriae) and *pef* (plasmid-encoded fimbriae). However, analysis has shown that the genomes of the serotypes contain different combinations of fimbrial gene sequences (Townsend *et al.*, 2001). Also, transcription of some of the fimbrial operons of *S*. Typhimurium is regulated by phase variation (Nicholson & David, 2000; Norris *et al.*, 1998b; Swenson & Clegg, 1992). Fimbrial proteins are known to be antigenic and are involved in eliciting host adaptive immunity during infection. Therefore, these characteristics probably enable *Salmonellae* of different serotypes to evade the host adaptive immune response, because otherwise, although they express O antigen polymorphism, possession of the same fimbrial proteins would enable the host to acquire cross-immunity (reviewed by Humphries *et al.*, 2001 and van der Woude & Baumler, 2004). The *S*. Typhimurium serotype also appears to utilise different fimbriae to target attachment to different cell types along the intestinal epithelium (Bäumler *et al.*, 1996c).

Type 1 fimbriae are the only adhesins that have been shown to be involved in the attachment to porcine IECs (Althouse *et al.*, 2003), a known site of *Salmonella* invasion. M cells of the ileal Peyer's patches are also considered a major site of invasion (Jepson & Clark, 2001; Jones *et al.*, 1994) and although not investigated in swine, the *lpf* fimbrial operon is known to mediate adhesion of *S*. Typhimurium to these cells in mice (Bäumler *et al.*, 1996b). Finally, a gene termed *invH* has been identified in *S*. Choleraesuis and *S.* Typhimurium and been shown to encode the InvH protein which is expressed in the bacterial membrane. Mutagenesis experiments showed that this gene is necessary for efficient adherence of *Salmonella* to cultured epithelial cells (Althouse *et al.*, 2003).

1.8.2 Invasion

Some interactions with host cells result in irreversible "docking" of the *Salmonella*, which commits the bacteria to invasion (Lara-Tejero & Galán, 2009; Misselwitz *et al.*, 2011b). *Salmonella* invade IECs and M cells to gain access to the underlying gut-associated lymphoid tissue (GALT). The GALT is highly organised and

constitutes the largest mass of immune cells in the body, notably B and T lymphocytes, which are activated on interaction with intestinal antigens (Mowat & Viney, 1997). It includes the Peyer's patches, appendix and isolated lymphoid follicles. Reed *et al*. (1986) conducted pig infection experiments and found *S.* Typhimurium had invaded porcine IECs and mesenteric lymph nodes 2 hours after oral infection. Another study involving porcine ileal loops showed that *S*. Typhimurium exhibited invasion of M cells, IECs and goblet cells as early as 10 minutes post-inoculation (Meyerholz & Stabel, 2003).

1.8.2.1 Salmonella *Pathogenicity Island 1*

Invasion is facilitated by the T3SS encoded within a region of the chromosome known as SPI-1. The T3SS machinery consists of a complex of proteins that span the inner and outer bacterial membranes, in addition to some cytoplasmic components. This contact-dependent secretion system acts as a needle-like complex whereby it injects proteins into the host cell cytosol (Collazo & Galán, 1997a; Cornelis, 2006). The proteins directly or indirectly stimulate signal transduction pathways that illicit a plethora of responses from the host cell. These responses include activation of transcription factors and membrane ruffling due to actin cytoskeleton rearrangements, which results in the uptake of the bacteria into the usually non-phagocytic host cell by macropinocytosis (Francis *et al*., 1993). The signalling events triggered by the exported *Salmonella* proteins occur due to direct engagement with the signalling machinery within the host cell (Hardt *et al*., 1998a), which is unlike other bacterial pathogens that induce signalling events by engaging cell surface receptors.

Target proteins secreted by the T3SS with effector functions in the host cell include SipA, SipB and SipC, which are encoded by genes located on the SPI-1 (Kaniga *et al*., 1995a; Kaniga *et al*., 1995b). SipB, SipC and SipD form a complex within the host cell membrane that is required for translocation of other effectors into the eukaryotic cell cytoplasm (Collazo & Galán, 1997b; Galyov *et al*., 1997; Wood *et al*., 1996). Along with SipA the remaining effectors that have been shown to contribute to *S.* Typhimurium invasion of epithelial cells *in vitro* include SopA, SopB, SopD and SopE2 (Bakshi *et al*., 2000; Raffatellu *et al*., 2005; Stender *et al*., 2000; Wood *et al*., 2000), which are encoded by genes found elsewhere in the Typhimurium chromosome. Regulation of the SPI-1 T3SS involves a complex feed-forward loop

in which regulators HilC, HilD and RtsA control expression of *hilA,* with HilD at the top of the hierarchy (Ellermeier & Slauch, 2007). HilA (hyperinvasion locus) is the central regulator of SPI-1 in that it is known to bind directly to promoters and activate the operons controlling the genes encoding all the components required for a functional T3SS (Lostroh & Lee, 2001). This regulatory system senses various environmental parameters and consequently the SPI-1 system is specifically induced when *Salmonella* is in the small intestine (Ellermeier & Slauch, 2007).

1.8.2.2 Salmonella Pathogenicity Island 2

The invading bacteria are taken up into unique loose-fitting vacuoles, known as *Salmonella*-containing vacuoles (SCV), unlike with receptor-mediated phagocytosis, which encapsulates particles into tight-fitting phagosomes derived from the plasma membrane (Brumell *et al.*, 1999). It is at this point that another T3SS encoded within the *Salmonella* genome, on SPI-2, comes into action. The SPI-2 T3SS is primarily responsible for driving maturation of the SCV, within which bacterial survival and proliferation is facilitated (Hensel, 2000), as well as translocating effector proteins across the SCV that control interactions between the vacuolar membrane and intracellular membrane compartments (Haraga *et al.*, 2008; Waterman & Holden, 2003). *Salmonella* survival and proliferation, moderated by SPI-2, occurs within IECs and, more importantly, within macrophages, dendritic cells and neutrophils (Abrahams & Hensel, 2006). SPI-2 function also disables host antimicrobial defence mechanisms, such as oxidative killing (Kuhle & Hensel, 2004; Waterman & Holden, 2003) and the overall outcome of these interferences is the facilitation of systemic spread of infection and modulation of host immune responses.

1.8.2.3 Coordination of gene expression

The genes encoded by SPI-2 are induced intracellularly, whereas SPI-1 genes are induced prior to internalisation of the bacterium (Eriksson *et al.*, 2003). Contrary to previous belief, however, further work has suggested that some of the genes encoded on the SPI-1 might be induced intracellularly and be involved in early intracellular pathogenesis given that there is a time lag between internalisation and SPI-2-associated protein synthesis (Knodler & Steele-Mortimer, 2003; Pfeifer *et al.*, 1999). Steele-Mortimer *et al.* (2002) showed that internalised bacteria with mutations in SPI-1 genes were unable to replicate inside cultured epithelial cells,

which indicates that SPI-1 effectors are essential for this process. The same study also describes a role for SPI-1 in SCV biogenesis. Similarly, after invasion, accumulation of cholesterol by the SCV occurs and appears to require bacterial effector protein delivery regulated by SPI-1, independently of SPI-2, at least in the early stages post-invasion (Garner *et al.*, 2002). Through the use of signature-tagged mutagenesis (STM), genes required by *S.* Typhimurium to colonise porcine intestines were identified (Carnell *et al.*, 2007). Of the 1045 signature-tagged mutants screened following oral inoculation of pigs, 119 attenuating mutations were identified, a large number of which were located on SPI-1 and SPI-2, which confirms the important roles of their associated T3SSs in intestinal colonisation in swine.

Coordination of gene expression during invasion of the intestinal epithelium involves, at least in some part, regulatory cross talk between the systems involved, as characterised by Saini *et al*. (2010). These authors identified that the flagella, SPI-1 T3SS and type 1 fimbriae systems are expressed sequentially during *in vitro* growth and that although this transcriptional hierarchy is controlled predominantly by external factors, specifically growth phase of the bacterial cell (Ernst *et al.*, 1990; Schmidt *et al.*, 2008), cross talk between the three systems is necessary for ensuring that expression of each of the systems is mutually exclusive. The importance of this becomes apparent when considering that, for example, expression of the flagellar genes enhances transcription of SPI-1 genes and therefore this regulation ensures that only actively motile cells attempt to invade host cells.

1.9 Host immune response

1.9.1 Intestinal epithelial cells

A single layer of epithelial cells lines the entire gastrointestinal tract from the stomach to the colon. The basal lamina separates these cells from the underlying *lamina propria*, which consists of smooth muscle cells, blood vessels and lymphatics. IECs are exposed to a variety of microbes within the intestinal tract including enteropathogens and commensal organisms. Differentiation between harmful and harmless microbes is vital in the maintenance of gut health; therefore IECs must respond appropriately to the differing antigenic stimuli. The gut mucosa has the ability to mount a protective immune response against harmful pathogens,

whilst remaining tolerant of harmless dietary antigens and microbial flora. Oswald (2006) reviewed the role of IECs in the innate immune defence of the intestine in pigs, including those features involved in direct inhibition of bacterial colonisation and those involved in interaction with components of the underlying GALT. The GALT is a site where B and T lymphocytes differentiate and defend, following interaction with luminal antigens that have breached the IEC monolayer.

1.9.1.1 Direct

One of the direct functions of the IEC layer is as a physical barrier between the contents of the gut lumen and the underlying tissues. Tight junctions and other intercellular structures contribute to the maintenance of the barrier (Gumbiner, 1993), the strength of which can be determined by measuring the trans-epithelial electrical resistance (TEER) of epithelial monolayers. Another direct function of IECs in mucosal defence is to secrete antimicrobial peptides, such as defensins and cathelicidins, which disrupt the integrity of microbial membranes. The majority of antimicrobial peptides identified in pigs belong to the cathelicidin family (Zhang *et al*., 2000); however, 12 porcine β-defensins (pBDs) have also been identified (Sang *et al*., 2006). Two of these, pBD-1 and pBD-2, have been shown to be expressed constitutively in the small intestine of pigs (Veldhuizen *et al*., 2007). However, there is conflicting evidence regarding the pattern of expression of these peptides in response to stimulation with *Salmonella.* One study reported upregulation of pBD-1 and pBD-2 in IPEC-J2 cells in response to infection with live *S*. Typhimurium, but not *S*. Choleraesuis (Veldhuizen *et al*., 2009), whereas in another study pBD-1 was not upregulated in response to *in vivo* stimulation of pigs with *S*. Typhimurium (Zhang *et al*., 1999a).

1.9.1.2 Indirect

IECs are indirectly involved in protecting the host against pathogens of the gut through their ability to secrete and respond to chemokines and cytokines. Secretion of these factors results in the recruitment of macrophages, neutrophils and lymphocytes by chemotaxis to the *lamina propria*, initiating both the innate and adaptive immune responses (Eckmann *et al*., 1993; Jung *et al*., 1995; Maaser & Kagnoff, 2002). The secretion of cytokines and chemokines is dependent on IEC detection and recognition of bacteria and bacterial products by Toll-like receptors (Philpott *et al*., 2001; Takeda & Akira, 2003). Stimulation of TLRs initiates a signal

transduction pathway, with downstream activation of nuclear factor (NF)-κB, which acts as a central regulator of the transcription of proinflammatory cytokines (Elewaut *et al.*, 1999). IECs also express chemokine receptors at their apical surface enabling them to respond to chemokines secreted by themselves or by other immune cells (Dwinell *et al.*, 1999). For example, IECs have been shown to respond to interleukin (IL)-1 via an IL-1 receptor by secreting IL-6 and IL-8 (Jung *et al.*, 1995; McGee *et al.*, 1993; Schuerer-Maly *et al.*, 1994), enhancing expression of mRNA for transforming growth factor-β (Dignass & Podolsky, 1993) and secreting acute phase response proteins (Molmenti *et al.*, 1993). In another study, stimulation of human IECs with tumour necrosis factor (TNF)-α or IL-6 resulted in an increase in intracellular adenosine triphosphate (ATP) levels, which may enable IECs to withstand heightened exposure to inflammatory mediators (Fukushima *et al.*, 1999). Taken together, it is clear that IECs are involved in mediating mucosal immune homeostasis through cross talk between themselves and other surrounding leukocytes (reviewed by Shaykhiev and Bals, 2007).

1.9.2 Toll-like receptors

TLRs are a family of homologous type 1 transmembrane proteins, most of which are expressed on the surface of leukocytes and epithelial cells. They belong to the group of molecules termed "pattern-recognition receptors (PRRs)" because of their specificity for pathogen-associated molecular patterns (PAMPs), which are conserved molecular structures shared by many different microorganisms. To date, 13 mammalian TLRs have been identified and the 10 that have been identified in humans have also been fully cloned in pigs (Uenishi & Shinkai, 2009). TLR molecules have a cytoplasmic domain homologous to that of the IL-1 receptor family and is so called Toll/IL-1 receptor (TIR) cytoplasmic domain. The TIR is highly conserved among TLR molecules. However, TLR molecules differ from IL-1 receptor proteins in that they have an extracellular region consisting of a leucine-rich repeat (LRR) domain (Medzhitov *et al.*, 1997). The LRR domain of TLRs forms a specific conformation, which provides a binding surface for specific pathogenic ligands (Bell *et al.*, 2003). The binding of a PAMP to a TLR induces a conformational change that enables the recruitment of myeloid differentiation factor 88 (MyD88). MyD88 is a 35 kDa adaptor protein that contains three functional domains: an amino-terminal death domain, an intermediate domain and a carboxyl-terminal TIR (Lord *et al.*, 1990). The carboxyl-terminal TIR domain of MyD88

interacts with the cytoplasmic TIR domain of the Toll protein and the amino-terminal death domain of MyD88 binds other proteins to the receptor complex, thus making it an essential component of the signal transduction pathway (Kopp & Medzhitov, 1999; Medzhitov *et al.*, 1998). There also exists a MyD88-independent signalling pathway due to the presence of MyD88 homologues, which is used preferentially for some TLRs (Alexopoulou *et al.*, 2001), or in conjunction with the MyD88-dependent pathway for other TLRs (Kawai *et al.*, 1999). A number of downstream targets are involved in the signal transduction of both pathways, which culminate in phosphorylation and degradation of the inhibitor of kappa B (IκB) allowing activation of the transcription factor NF-κB. Consequently, translocation of NF-κB to the nucleus results in the expression of genes encoding proinflammatory cytokines, which initiates neutrophil migration to the mucosa (Rothkötter *et al.*, 1999). TLRs are therefore considered to be key drivers of host innate immune defence mechanisms against pathogens and serve as a link between the innate and adaptive immune responses.

The presence of two signal transduction pathways and multiple ligands and receptors adds specificity to the TLR signalling network, ensuring that appropriate immune responses are elicited according to the pathogen involved. The respective ligands for each of the known TLRs are outlined in Table 1.2. Briefly, there are two main groups of TLRs: 3, 7, 8 and 9 collectively respond to nucleic acids of bacteria and viruses, stimulating the production of antiviral type-1 interferons (Uematsu & Akira, 2007), while TLRs 1, 2, 4, 5, 6, 10 and 11 recognise outer membrane components of bacteria, fungi and protozoans. Whereas most TLRs signal as homodimers, TLR-1, TLR-6 and TLR-10 all mediate responses in cooperation with TLR-2 as heterodimers (Ozinsky *et al.*, 2000).

The expression of TLRs by various porcine cells and tissues has been characterised in a number of studies (Ålvarez *et al.*, 2006; Muneta *et al.*, 2003; Raymond & Wilkie, 2005; Shimosato *et al.*, 2003; Shimosato *et al.*, 2005; Thomas *et al.*, 2006; Tohno *et al.*, 2005). Analysis of tissue samples (tonsil, jejunum, ileum, colon, mesenteric lymph node, spleen and liver) from healthy pigs demonstrated that TLRs 2, 4, 5 and 9 were expressed in all tissues, with expression generally being greater in all other tissues compared to the liver and the most prominent relative expression occurring in the colon for TLR-2, TLR-4 and especially TLR-5 (Burkey *et al.*, 2007). In the same study, TLR mRNA levels following *in vivo* exposure to *S.* Typhimurium and *S.*

Choleraesuis were analysed, with both TLR-5 and TLR-9 being upregulated in the jejunum in response to both serotypes. Other studies have also attempted to characterise TLR involvement in *Salmonella* infection. For example, in a porcine gut loop model, Meurens *et al.* (2009) characterised the early immune response to *S*. Typhimurium infection in gut tissue. TLR-2 and TLR-4 mRNA expression was upregulated, yet surprisingly TLR-5 expression was downregulated. Arce *et al.* (2010) quantified the relative expression of TLRs 1, 2, 3, 4, 6, 8, 9 and 10 in two porcine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from *S.* Typhimurium. All of the TLRs were shown to be expressed in both cell lines and although there were differences in TLR gene expression between the two lines, TLR-2 and TLR-4 were up-regulated by both IPEC-J2 and IPI-2I in response to *Salmonella* LPS. Interestingly, the IPI-2I line, which is of ileal origin, expressed most of the TLRs more highly than the jejunal IPEC-J2 line, which could be linked to the fact that the ileum is a preferential site for *Salmonella* invasion compared to the jejunum (Boyen *et al.*, 2008a; Darwin & Miller, 1999) and therefore requires a greater innate immune response.

1.9.3 Inflammation

Zeng *et al*. (2003) identified that although the SPI-1 T3SS is required for invasion of epithelial cells, it is not essential for the host cells to detect the pathogenic organism or to mount a proinflammatory response, as shown by SPI-1-deficient mutant strains exhibiting only minimally reduced proinflammatory potential, especially compared to aflagellate strains. Santos *et al*. (2009) reviewed how *S*. Typhimurium initiates acute intestinal inflammation through release of proinflammatory cytokines, such as IL-18 and IL-23, following direct interaction with host cells. These cytokines facilitate amplification of mucosal barrier function by stimulating T cells to produce interferon (IFN)-γ, IL-17 and IL-22 (Godinez *et al.*, 2008). An example of a host-pathogen interaction that results in the production of proinflammatory cytokines is the detection of bacterial products by host cell receptors. In addition to playing an important role in colonisation of the intestinal mucosa, flagellin has been identified as one such bacterial product, or PAMP, that induces production and release of the chemokine IL-8 from intestinal epithelial cells (Gewirtz *et al.*, 2001b). This response is dependent on the translocation of flagellin to the basolateral surface of the epithelial cells where it acts as a stimulatory ligand for TLR-5 (Gewirtz *et al.*, 2001a). The significance of the need to translocate flagellin to the basolateral surface is

presumably to avoid TLR-5 ligation by the flagellin of normal intestinal flora (Salazar-Gonzalez & McSorley, 2005).

Table 1.2 Example ligands of mammalian Toll-like receptors.

TLR	Ligand	Reference
TLR-1 (in association with TLR-2 as a heterodimer)	Bacterial lipopeptides	Wyllie *et al.*, 2000 Takeuchi *et al.*, 2002
TLR-2	Peptidoglycan and lipopeptides of Gram-positive bacteria	Schwandner *et al.*, 1999 Takeuchi *et al.*, 1999
TLR-3	Double-stranded RNA of viruses	Alexopoulou *et al.*, 2001
TLR-4	LPS of Gram-negative bacteria	Poltorak *et al.*, 1998
TLR-5	Bacterial flagellin	Hayashi *et al.*, 2001
TLR-6 (in association with TLR-2 as a heterodimer)	Bacterial lipopeptides	Ozinsky *et al.*, 2000 Takeuchi *et al.*, 2001
TLR-7 & TLR-8	Synthetic compounds; ssRNA	Hemmi *et al.*, 2002 Jurk *et al.*, 2002 Heil *et al.*, 2004
TLR-9	CpG motifs of bacterial DNA	Hemmi *et al.*, 2000
TLR-10 (in association with TLR-2 as a heterodimer)	Bacterial lipopeptides	Guan *et al.*, 2010
TLR-11	Uropathogenic *E. coli*; profilin-like protein of *T. gondii*	Zhang *et al.*, 2004 Yarovinsky *et al.*, 2005

Basolateral release of IL-8 from IECs results in recruitment of neutrophils to the *lamina propria*, while apical release of hepoxilin A3, another chemoattractant, directs movement of the neutrophils across the intestinal epithelium into the gut lumen (Gewirtz *et al.*, 1999; McCormick *et al.*, 1998). The recruited neutrophils play an important role in initiating and orchestrating the early inflammatory events that occur after *Salmonella* infection, but their activity can be both beneficial and detrimental to

the host. The migration of neutrophils results in disruption of the integrity of the epithelium and an increase in vascular permeability, which causes transepithelial fluxes of ions and leakage of extravascular fluids into the intestinal lumen (Eckmann *et al.*, 1997; Zhang *et al.*, 2003a). These events are instrumental in causing the pathology of enterocolitis (inflammation of the digestive tract), including diarrhoea. However, *Salmonella* can be killed by the attracted neutrophils. The presence of high numbers of neutrophils in the porcine gut enables clearing of the infection (Foster *et al.*, 2003; Foster *et al.*, 2005) and the acute inflammatory diarrhoea limits systemic spread of the bacteria.

Systemic spread of *Salmonella* in pigs is usually associated with *S.* Choleraesuis and is not well documented for *S.* Typhimurium infection. Paulin *et al.* (2007) characterised the virulence of *S.* Choleraesuis and *S.* Typhimurium during infection of a porcine ligated ileal loop. It was observed that *S.* Typhimurium virulence was associated with rapid intracellular replication in the intestinal mucosa and greater induction of proinflammatory cytokines, whereas *S.* Choleraesuis virulence involved slower replication and enhanced persistence in the intestinal mesenteric lymph nodes. As previously described, the rapid induction of proinflammatory responses by Typhimurium enables pigs to limit infection to the intestines, however the slow replication of Choleraesuis presumably enables it to evade the host innate immune response and disseminate systemically by stealth. The significance of gut inflammation during acute enteric infection with *S.* Typhimurium has already been briefly discussed; damage to the gut mucosa during inflammation results in increased availability of nutrients, which can be accessed by *Salmonella* through flagella-mediated movement and utilised for faster replication (Stecher *et al.*, 2008). Winter *et al.* (2010) investigated this further and demonstrated that during respiratory burst of phagocytes recruited during inflammation, thiosulphate is oxidised to tetrathionate, which can be fermented by *S.* Typhimurium for growth. This gives *S.* Typhimurium a selective advantage over competing microbes in the gut, which are unable to ferment tetrathionate. Tetrathionate-facilitated *Salmonella* growth promotion was not observed in the non-inflamed gut in the same study. This demonstrates how *S.* Typhimurium has evolved to exploit host immune responses for its own pathogenesis; enrichment for *S.* Typhimurium during growth in the inflamed gut leads to increased transmission by the faecal-oral route (Lawley *et al.*, 2008).

1.9.4 Apoptosis

In addition to IL-8, other chemokines are released from cells of the porcine gastrointestinal tract in response to *Salmonella* invasion, including IL-1, IL-6 and TNF-α (Cho & Chae, 2003; Hyland *et al.*, 2006a; Hyland *et al.*, 2006b; Trebichavsky *et al.*, 2003). TNF-α is responsible for activating and regulating neutrophils, mononuclear phagocytes and other immune cell types (Jung *et al.*, 1995). It also has an important role in regulating the later apoptotic response of intestinal epithelial cells to intracellular and replicating bacteria (Kim *et al.*, 1998). Apoptosis of IECs under normal pathogen-free conditions is a means of maintaining a balance in the number of cells present at the intestinal mucosa, necessary because of constant cell proliferation and occurs spontaneously without causing inflammation (Hockenbery, 1995). Following apoptosis, cellular debris is engulfed by adjacent epithelial cells and sub-epithelial macrophages and shed into the lumen of the gut (Hall *et al.*, 1994). However, due to an upregulation of TNF-α by IECs following infection with enteroinvasive bacteria, including *Salmonella*, increased levels of apoptosis occur. The stimulation of apoptosis in these cells occurs after a delay because bacterial invasion also activates NF-κB, a transcription factor that suppresses cell death signals, thus temporarily promoting cell survival (Dyer *et al.*, 1993; Elewaut *et al.*, 1999). This delayed onset of apoptosis benefits the epithelial cells, in that it allows sufficient time for generation of proinflammatory response signals to enhance host survival, but also benefits the bacteria, in that it provides more time for the invading bacteria to replicate before invading further into the mucosa (Kim *et al.*, 1998). Rapid upregulation of inducible nitric oxide synthase (iNOS) expression and production of nitric oxide is another characteristic of the early response of human colon epithelial cells to bacterial invasion (Witthoft *et al.*, 1998) and has been linked to increased apoptotic cell death (Kim *et al.*, 1998; Sandoval *et al.*, 1995). However, whether porcine intestinal epithelial cells exhibit these mechanisms is not clear, although it has been suggested that NOS is not inducible in swine (Pampusch *et al.*, 1998).

1.9.5 Pyroptosis

Besides neutrophils, macrophages are also recruited to the infected intestinal mucosa and GALT, where they play an important role in controlling and clearing *Salmonella*. Macrophages are able to destroy the bacteria by phagocytosis like

neutrophils but the interaction between the bacteria and macrophages involves a struggle between the macrophage's pathogen killing mechanisms and the bacteria's survival mechanisms. The result of this struggle ultimately determines the disease pathogenesis (Lindgren *et al.*, 1996; Wijburg *et al.*, 2000). A unique characteristic of the interaction between *Salmonella* and macrophages is the stimulation of macrophages to undergo a proinflammatory cell death, termed "pyroptosis" (Cookson & Brennan, 2001; Fink & Cookson, 2007). *S.* Typhimurium, *S.* Typhi, *S.* Dublin and *S.* Gallinarum have all been shown to trigger pyroptosis in murine macrophages, showing that it is a property not exclusive to broad host range strains (Chen *et al.*, 1996b). The pyroptotic cell death triggered in macrophages is uniquely dependent on caspase-1, an enzyme that activates precursors of the inflammatory cytokines IL-1β and IL-18 (Fantuzzi & Dinarello, 1999) and results in rapid macrophage cell lysis and release of proinflammatory cytoplasmic contents (Brennan & Cookson, 2000; Hersh *et al.*, 1999). The significance of the release of IL-1β and IL-18 upon lysis of the macrophage is that they are primary drivers of acute and chronic inflammation (Braddock *et al.*, 2004), highlighting the contribution of pyroptosis to the tissue destruction and inflammation that is characteristic of both typhoid fever and non-typhoidal *Salmonella* infection. IL-18 induces production of IFN-γ, a well-studied cytokine that is expressed by T cells and natural killer (NK) cells, whose main function is to initiate bactericidal activity in macrophages (Kagaya *et al.*, 1989). Neutralisation of IL-18 results in increased bacterial load in the spleen and liver of mice and decreased host survival (Dybing *et al.*, 1999; Mastroeni *et al.*, 1999).

The SPI-1 T3SS and flagellin are both involved in triggering pyroptosis, evidenced by the fact that aflagellate *Salmonella* are not cytotoxic to macrophages (Franchi *et al.*, 2006; Miao *et al.*, 2006; Miao *et al.*, 2010), neither are strains containing mutations in the genes encoding the SPI-1 T3SS (Chen *et al.*, 1996b; Monack *et al.*, 1996) and the regulator gene for SPI-1, *hilA* (Lundberg *et al.*, 1999). Monack and co-workers (1996) identified that membrane ruffling and bacterial internalisation is required for activation of macrophage cell death. They also found that *Salmonella* strains that are defective in intracellular replication are still capable of triggering pyroptosis, presumably because replicative ability is associated with the SPI-2 T3SS, which is unlikely to become active before cell death occurs. Conversely, the aforementioned non-inflammatory apoptosis triggered in *Salmonella*-infected IECs is

activated by the executioner protein caspase-3, whose activation is associated with expression of genes located on the *spv* locus and SPI-2 (Paesold *et al.*, 2002). Another key difference between the two types of cell death is that during apoptosis the plasma membrane of the host cell remains intact, which is a critical feature for preventing release of the inflammatory intracellular contents. Apoptotic bodies bound by plasma membrane are phagocytised by other macrophages, which enables degradation of cellular components without causing damage to surrounding tissues (Savill *et al.*, 2002).

1.9.6 Delayed macrophage death

Although rapid macrophage death by pyroptosis is crucial for inflammation and tissue damage, it also hinders systemic spread of *Salmonella*, thus limiting the disease to a local infection. However, it has been demonstrated that *Salmonella* is capable of downregulating SPI-1 T3SS and flagellin expression in order to avoid rapid pyroptosis (Cummings *et al.*, 2005; Lundberg *et al.*, 1999). Delaying host cell death enables persistence and proliferation of the bacteria within macrophages, which facilitates systemic bacterial infection through dissemination of bacteria to internal organs. Once replication and dissemination has been achieved, delayed macrophage death is triggered independently of SPI-1, by a SPI-2 T3SS-associated mechanism (Monack *et al.*, 2001; van der Velden *et al.*, 2000). Delayed SPI-2-dependent macrophage death is similar to SPI-1-dependent death in that it too is primarily mediated by caspase-1 and involves production of IL-1β and IL-18 and cell lysis (Monack *et al.*, 2001). This program of delayed macrophage cytotoxicity has been described in mice infected with the broad-host range serotype *S.* Typhimurium (van der Velden *et al.*, 2000), as well as in humans infected with the host-restricted serotype *S.* Typhi (Monack *et al.*, 2001), both of which are associated with systemic disease in the respective host. In humans and mice, systemic spread of *Salmonella* within macrophages occurs via the blood stream and/or lymphatic fluids and can result in the colonisation of the mesenteric lymph nodes, spleen and liver, accompanied by pronounced systemic and local immune responses.

Surprisingly, there is evidence that *S.* Typhimurium also causes delayed pyroptosis in bovine macrophages (Santos *et al.*, 2001) and in human macrophages (Browne *et al.*, 2002), despite this serotype being associated with localised gastroenteritis rather than systemic disease in these hosts. During infection of porcine alveolar

macrophages, *S.* Typhimurium and *S*. Dublin induced damage to the plasma membrane, but *S*. Choleraesuis did not, suggesting that the latter causes delayed pyroptosis, which is consistent with its ability to cause systemic disease in pigs (Watson *et al.*, 2000). Systemic infection of pigs with *S.* Typhimurium is not well-documented, and although in one study germ-free piglets experienced prominent systemic immune responses as a result of penetration of extra-intestinal organs by a specific strain of *S*. Typhimurium (Dlabac *et al*., 1997), another study noted that shortly after experimental inoculation, sporadic Typhimurium bacteria present in the liver and spleen of pigs did not appear to replicate and were cleared from these organs within a few days post-inoculation (Boyen *et al.*, 2009).

1.9.7 Host-specific immune response

It is important to note that differences exist between the immune systems of swine, humans and mice and, as a result, infection with *Salmonella* and other pathogens can have markedly different effects depending on the host species (reviewed by Scharek and Tedin, 2007). For example, unlike the discrete Peyer's patches (PPs) found in many species, juvenile pigs have an ileal PP that is distal, continuous and very long and its cellular population is composed of more than 80% B cells (Makala *et al*., 2002). However, like the discrete PPs, it is covered by a follicle-associated epithelium containing M cells, which enable antigen sampling by taking up antigens from the gut lumen and passing them to the underlying antigen presenting cells. Different patterns of lectin glycoprotein binding displayed on the cell surface of M cells are exhibited by different species and this is potentially responsible for the host adaptation of some *Salmonella* serotypes (Scharek and Tedin, 2007). Another contrasting feature of the porcine immune system is the lack of expression of major histocompatibility complex (MHC) class II on intestinal epithelial cells, meaning that they do not present antigens, unlike human and murine IECs. Instead, these cells in pigs produce IL-8 as previously described; an activity not displayed by IECs of mice. Also, production of α-defensins, usually the most abundant antimicrobial peptides in mammals, has not been identified in swine, and expression of the porcine β-defensin does not seem to be upregulated in response to *S.* Typhimurium infection, suggesting that it has a maintenance role in the control of mucosa-associated bacteria (Zhang *et al*., 1999b). Finally, several differences in the phenotypes of porcine immune cells have been identified, such as the existence of $CD4^+/CD8^+$ double positive T cells, not described in mice or humans. These double positive

lymphocytes exhibit properties of mature memory T cells, inducible by stimulation with recall antigen (Zuckermann & Husmann, 1996). These findings highlight the dangers of comparing intestinal models of *Salmonella* infection in mice, to those of humans and pigs.

The conditions that favour subclinical *Salmonella* infection and the characteristic "carrier" state in pigs are not well understood, however distinctive features of *Salmonella* infection in swine, might be explained in an immunological context. For example, high bacterial loads in the tonsils of pigs is usual during *Salmonella* infection and viable bacteria can be recovered from this site in high numbers long after serology and faecal and intestinal bacteriology point towards effective clearing of the bacteria (Wood *et al.*, 1989). The significance of this is that the tonsils represent an important location site of antigen presenting cells including dendritic cells, lymphocytes and macrophages, the presence of which may enable *Salmonella* to persist in this lymphoid organ. Studies have shown that *Salmonella* can invade or be taken up by B cells (Ugrinovic *et al.*, 2003; Verjans *et al.*, 1994) and these host cells possibly constitute an immunologically protected niche for *Salmonella* and other intracellular pathogens, since the bacteria can suppress the MHC presentation pathways of human and murine B cells (Mitchell *et al.*, 2004; Qimron *et al.*, 2004) and murine dendritic cells (Cheminay *et al.*, 2005; Tobar *et al.*, 2006), thus preventing activation of $CD8^+$ cytotoxic T cells and hindering activation of the adaptive immune response. Similar studies, however, have yet to be conducted with porcine immune cells. Differences between the number (Yang *et al.*, 1996) and phenotype (Denyer *et al.*, 2006) of porcine, human and murine γδ T cells might also be a contributing factor towards the alternative course of *Salmonella* infection in swine, especially as these cells are known to play an important role in the clearance of the bacteria in mice (Davies *et al.*, 2004a). Further immunological reasons for the pathophysiology of *Salmonella* infection of swine might also be routed in the production system(s) of pigs. Scharek and Tedin (2007) suggest that typical pig production features such as early weaning, feeding habits, pelleted feed regimes, high fibre diets, rearing conditions and sterilisation methods are likely to influence the status of the immune system in a way that contributes towards persistent latent infections.

1.10 Aims

Monophasic variants of *Salmonella* are a growing human and animal health concern. The reason for their emergence and consequent spread from commercial pig populations is not known. The selective pressure driving monophasic expression of flagellar antigen and the effect of this phenotype on pathogenesis in the porcine host is also unknown. The intention of this thesis is to characterise isolates of monophasic *Salmonella* phage type DT193 and to discuss the findings in relation to results from biphasic *S.* Typhimurium isolates of the same phage type. The specific aims are to:

- Elucidate aspects of the genotype and phenotype, including motility, biofilm formation and virulence protein secretion, of *Salmonella* DT193 isolates.
- Assess the gut colonisation potential of *Salmonella* DT193 isolates by quantifying their adhesion to and invasion of mammalian host cells.
- Evaluate the early immune response of porcine intestinal epithelial cells to *Salmonella* DT193 infection *in vitro*, by determining stimulation of proinflammatory and apoptotic pathways.

2 Phenotypic Characterisation of DT193

2.1 Introduction

The evolution of *Salmonella* has been punctuated by the emergence of epidemic and multi-drug resistant strains (reviewed by Rabsch *et al.*, 2001). Examples include *S.* Enteritidis, which is associated with laying hens and *S.* Typhimurium DT104, associated primarily with cattle. These strains pose a threat to public health because of their ability to spread widely and rapidly and their tendency to persist in food-producing animal populations. Over the past two decades, monophasic strains of *Salmonella* (serotype 4,[5],12:i:-) have demonstrated rapid worldwide emergence and in Europe the main reservoir species appears to be pigs (Hopkins *et al.*, 2010). In Europe, monophasic strains are now the third most common serotype of *Salmonella* isolated from pigs and pig meat (Anon., 2013c), and the third most common from humans (Anon., 2014a). In a recently published expert opinion, monophasic strains of *Salmonella* were deemed to be of increasing importance in Europe due to their causing of a substantial number of infections in both humans and food-producing animals (Anon., 2010d).

Salmonella serotype 4,[5],12:i:- isolates are described as "monophasic" because they do not express the *fljB* gene for second phase flagellar antigen and instead express only first phase flagella, coded for by the *fliC* gene. As a result of this genotype, their antigenic formula is designated 4,[5],12:i:-. Strains lacking expression of the first phase flagellar antigen, or lacking both phases do also exist, but they are uncommon. Various molecular techniques, including multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and multilocus variable number tandem repeat analysis (MLVA) have identified common genetic factors in *S.* Typhimurium and monophasic strains, suggesting that most isolates of emerging monophasic *Salmonella* are variants of *S.* Typhimurium (Achtman *et al.*, 2012; Alcaine *et al.*, 2006; Amavisit *et al.*, 2005; Bugarel *et al.*, 2012; Echeita *et al.*, 2001; Guerra *et al.*, 2000; Laorden *et al.*, 2010; Soyer *et al.*, 2009; Zamperini *et al.*, 2007). Furthermore, 4,[5],12:i:- isolates have been shown by molecular methods to have similar virulence gene repertoires and antimicrobial resistance cassettes to *S.* Typhimurium (Guerra *et al.*, 2004; Hauser *et al.*, 2010; Rodríguez *et al.*, 2008; Soyer

et al., 2009), suggesting that the public health risk posed by these strains is comparable to that of other epidemic *S*. Typhimurium strains.

In the past, *S*. Typhimurium was thought of as a clonal population, with most isolates existing as clones with worldwide distribution (Beltran *et al.*, 1988). Indeed, often multiple genotypic and phenotypic methods must be used together to discriminate between different phage types of *S*. Typhimurium (Gebreyes *et al.*, 2006). However, it has since been shown that there is considerable genetic diversity within the serotype as a result of mutation and recombination events (Lan *et al.*, 2009). In fact, studies investigating the clonality of epidemic strains, mostly *S.* Typhimurium DT104, have identified considerable molecular and antimicrobial resistance diversity among isolates of the same phage type (Farzan *et al.*, 2008; Gebreyes *et al.*, 2006; Liebana *et al.*, 2002; Poppe *et al.*, 2002). Such genetic heterogeneity has also been observed for *S*. Typhimurium phage type DT193 (Gebreyes *et al.*, 2006; Hampton *et al.*, 1995). Molecular typing of 4,[5],12:i:- isolates from around the world has revealed distinct phage types, antibiotic resistance types, PFGE profiles and MLVA patterns, suggesting that they evolved from *S*. Typhimurium through multiple independent emergence events (Hauser *et al.*, 2010; Soyer *et al.*, 2009). Despite this, the emergence of 4,[5],12:i:- in Europe has been dominated by just two major clonal lineages; the European clone harbouring chromosomally encoded tetra-resistance to ampicillin, streptomycin, sulphonamides and tetracyclines (R-type ASSuT; (Argüello *et al.*, 2014; Hauser *et al.*, 2010; Hopkins *et al.*, 2010; Lucarelli *et al.*, 2010) and the Spanish clone with plasmid-mediated resistance to multiple antimicrobial drugs (Echeita *et al.*, 1999; García *et al.*, 2011; Guerra *et al.*, 2001).

4,[5],12:i:- isolates can be phage typed according to the scheme of Anderson *et al.* (1977), with the majority of isolates in Europe typing to the recognised *S*. Typhimurium phage types DT193 and DT120 (Gallati *et al.*, 2013; Hauser *et al.*, 2010; Hopkins *et al.*, 2010). In the UK, *S*. Typhimurium DT193 is one of the phage types most frequently isolated from humans and retrospective analysis of DT193 isolated from patients in 2010 revealed that 52% were monophasic (Hopkins *et al.*, 2012). Monophasic strains of *Salmonella* isolated from pigs, pork and humans have a high level of homogeneity (García *et al.*, 2013; Hauser *et al.*, 2010), highlighting their likely zoonotic nature. *S*. Typhimurium DT193 and its 4,[5],12:i:- variants pose a significant threat to public health because pigs and pork are considered to

represent the second most important source of human *Salmonella* infection in Europe, with eggs and laying hens being the foremost (Pires & Hald, 2010; Pires *et al.*, 2010). Indeed, a 4,[5],12:i:- DT193 variant carrying ASSuT resistance caused two major human outbreaks in Luxembourg in 2006 (Mossong *et al.*, 2007) and large diffuse outbreaks in Germany around the same time (Trüpschuch *et al.*, 2010).

Analysis of 4,[5],12:i:- strains to date has largely been based on molecular typing and there has been no investigation of the pathogenicity of monophasic or biphasic DT193 isolates, except one study that investigated their ability to cause infection in poultry (Parsons *et al.*, 2013). Similarly, there have been only a small number of studies examining the phenotypic traits (apart from antimicrobial resistance) of this epidemic strain (Brunelle *et al.*, 2013; Rajtak *et al.*, 2012; Seixas *et al.*, 2014). It is necessary to document the phenotype of isolates in order to build a complete understanding of their virulence and epidemiology. Evidence from phenotypic studies of field isolates of *S*. Enteritidis has shown great phenotypic diversity including bacterial growth, ability to form biofilm, survival in egg albumen and virulence potential in poultry (Clavijo *et al.*, 2006; Shah *et al.*, 2011; Yim *et al.*, 2010), even within the same phage types and clonal lineages, despite this serotype being the most genetically homogenous of all the *Salmonella* serotypes (Hudson *et al.*, 2001; Liebana *et al.*, 2001; Porwollik *et al.*, 2005). Therefore, it is important to characterise the individual phenotypes of even highly clonal isolates, to expose any incongruencies and to gain clues about their virulence. To begin with, the growth characteristics, virulence gene profiles, flagellar features, motility, biofilm formation abilities and secreted protein profiles of *Salmonella* 4,[5],12:i:- DT193 and Typhimurium DT193 isolates were investigated.

2.2 Materials and Methods

2.2.1 *Salmonella* isolates

The isolates of bacteria used throughout this study are detailed in Table 2.1 and Table 2.4. The isolates of pig origin were kindly donated by the Animal Health Veterinary Laboratories Agency (Surrey, UK) who isolated them from pig farms in Great Britain.

Table 2.1 Field isolates of *Salmonella* used in this study and their antimicrobial resistance profile.

Isolate	Serotype	Phage type	Resistance profile	Origin
S01299	4,12:i:-	DT193	T, A, S, SU	Pig
S04327	4,5,12:i:-	DT193	T, A, CAZ, SXT, CM, CN, S, SU, CTX, APR	Pig
S03554	4,5,12:i:-	DT193	T	Pig
S00398	Typhimurium	DT193	NA, T, N, A, SXT, CM, CN, CIP, S, SU, APR	Pig
S01557	Typhimurium	DT193	T	Pig
L00168	Typhimurium	DT193	T, A, S, SU	Pig
4/74	Typhimurium	-	NA	Cattle*
P125109	Enteritidis	PT4	NA	Human/Chicken†

Abbreviations: T, tetracycline; A, ampicillin; S, streptomycin; SU, sulfamethoxazole; CAZ, ceftazidime, SXT, sulfamethoxazole with trimethoprim; CM, chloramphenicol; CN, gentamicin; CTX, cefotaxime; APR, apramycin; NA, nalidixic acid; N, neomycin; CIP, ciprofloxacin.

References: *Rankin & Taylor (1966; 2005); †Thomson *et al.*,(Thomson *et al.*, 2008).

2.2.2 Bacterial culture conditions

All *Salmonella* isolates were stored on Microbank™ cryoprotective beads (Pro-Lab Diagnostics Inc., UK) at -80°C. Using aseptic technique, isolates were streaked from frozen stocks onto nutrient agar and incubated aerobically for 24 h at 37°C. Stationary phase cultures were prepared by inoculating 10 ml standard Miller formulation Luria-Bertani (LB) broth with a few colonies from a nutrient agar plate using a sterile loop. Cultures were incubated overnight for 16-18 h at 37°C, 150 rpm in an orbital shaker. Late-logarithmic phase cultures were achieved by diluting stationary phase cultures 1:100 (v/v) into fresh LB broth followed by incubation at 37°C, 150 rpm for a further 3.5 hours.

2.2.3 Bacterial growth curves

A few colonies of *Salmonella* were picked with a sterile loop from nutrient agar plates and used to inoculate 30 ml LB broth in 75 cm^3 disposable polystyrene flasks. Cultures were incubated at 37°C, 150 rpm for up to 24 hours. Every hour, 1 ml of culture was collected into a cuvette for measurement of optical density at 600nm (OD_{600}) with a spectrophotometer. Sterile LB broth was used to blank the instrument. Samples were serially diluted and plated out to determine viable bacterial counts by the method of Miles *et al*. (1938). Briefly, serial dilutions were made in sterile phosphate-buffered saline (PBS) and 20 µl of each dilution were plated onto nutrient agar. Agar plates were incubated at 37°C for 20-24 h, after which the number of colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 60 colonies.

2.2.4 PCR

2.2.4.1 Virulotyping

Salmonella isolates were grown on nutrient agar plates for 20-24 h at 37°C. Crude boil lysate DNA samples were prepared by inoculating 500 µl sterile distilled water with 2-3 bacterial colonies. Preparations were boiled at 100°C for 10 minutes. Isolates were screened for the presence of 13 genes associated with virulence, the primers for which are listed in Table 2.2. Each 25 µl reaction mixture contained 1 µl DNA, 1 µl (100 pmol) each of the forward and reverse primers and 22 µl of 1.1x ReddyMix™ PCR Master Mix containing 1.5 mM $MgCl_2$ (Thermo Scientific, UK). PCR thermocycler conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension step of 72°C for 5 min (Hughes *et al.*, 2008; Zhang *et al.*, 2006).

2.2.4.2 Presence of flagella-associated genes

Additionally, the isolates were screened for the presence of genes involved in flagella formation and phase variation. Primers for *fliC*, *fljB*, *fljA* and *hin* were designed using the public software Primer-BLAST (Ye *et al.*, 2012) and the sequences are detailed in Table 2.3. Reaction mixtures and thermocycler conditions were as described in Section 2.2.4.1.

2.2.4.3 Agarose gel electrophoresis

PCR products were run by electrophoresis on an agarose gel. Stock (10x) Tris-acetate-EDTA (TAE) buffer was prepared by dissolving 48.5 g Tris base, 11.4 ml glacial acetic acid and 20 ml 0.5M EDTA in 1 litre dH_2O. Dilution of 10x TAE buffer 1:10 (v/v) in dH_2O gave a 1x working solution. 1.5% (w/v) gels were prepared by addition of 3.8 g agarose into 250 ml 1x TAE buffer, which was heated until the agarose had dissolved. After the molten solution had cooled slightly, 6.2 μl ethidium bromide was added and the mixture was poured into a gel cast with a multi-well comb. Once the gel had set, the comb was removed and 10 μl of each PCR product was loaded into the wells. A working solution of ΦX174 HaeIII digest DNA marker (Thermo Scientific, UK) was also loaded onto the gel for estimation of PCR product amplicon size. The gel was then transferred to a gel electrophoresis tank filled with (1x) TAE buffer and run at 120 V for 30-40 min. Finally, the DNA products were visualised under ultraviolet light in a transilluminator.

Table 2.2 PCR virulotyping primer sequences and gene functions.

Gene	Function	Primer Sequence (5' to 3')	Reference
prgH	SPI-1 T3SS apparatus	F: GCCCGAGCAGCCTGAGAAGTTAGAAA R: TGAAATGAGCGCCCCTTGAGCCAGTC	Skyberg *et al*., 2006
sopB	SPI-1 T3SS secreted effector protein	F: CGGACCGCCCAGCAACAAAACAAGAAGAAG R: TAGTGATGCCCGTTATGCGTCAGTGTATT	Skyberg *et al*., 2006
sopE	SPI-1 T3SS secreted effector protein	F: TCAGTTGGAATTGCTGTGGA R: TCCAAAAACAGGAAACCACAC	Hopkins & Threlfall, 2004
sopE2	SPI-1 T3SS secreted effector protein	F: TACTACCATCAGGAGGCATTC R: CCGCAGTACGTGACTAACATA	This study
invA	SPI-1 T3SS apparatus	F: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R: AGTTTCTCCCCCTCTTCATGCGTTACCC	Skyberg *et al*., 2006
sitC	Iron transport system	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC	Skyberg *et al*., 2006
spiC	SPI-2 T3SS secreted effector protein	F: CCTGGATAATGACTATTGAT R: AGTTTATGGTGATTGCGTAT	Hughes *et al*., 2008
sifA	SPI-2 T3SS secreted effector protein	F: TTTGCCGAACGCGCCCCCACACG R: GTTGCCTTTTCTTGCGCTTTCCACCCATCT	Skyberg *et al*., 2006
misL	SPI-3 autotransporter protein	F: GTCGGCGAATGCCGCGAATA R: GCGCTGTTAACGCTAATAGT	Hughes *et al*., 2008
orfL	SPI-4 autotransporter protein	F: GGAGTATCGATAAAGATGTT R: GCGCGTAACGTCAGAATCAA	Hughes *et al*., 2008
pipD	SPI-5 T3SS secreted effector protein	F: CGGCGATTCATGACTTTGAT R: CGTTATCATTCGGATCGTAA	Hughes *et al*., 2008
iroN	Iron acquisition	F: ACTGGCACGGCTCGCTGTCGCTCTAT R: CGCTTTACCGCCGTTCTGCCACTGC	Skyberg *et al*., 2006
pefA	Fimbria protein	F: GCGCCGCTCAGCCGAACCAG R: CAGCAGAAGCCCAGGAAACAGTG	Skyberg *et al*., 2006

Table 2.3 Primer sequences for flagella-associated genes.

Gene	Function	Primer Sequence (5' to 3')
fliC	Phase 1 flagella filament	F: CGCTATCGAGCGTCTGTCTT R: GTCACCTCACCGTTCGTCTT
fljB	Phase 2 flagella filament	F: TACGATGAAGCGACAGGAGC R: TCGCGTAGTCGGAATCTTCG
fljA	*fliC* repressor	F: CTACGGGCGAGAAGCTGAAA R: TCCTGCTCACCCAGTCAAAC
hin	Inversion recombinase	F: GCGGCTTGCCGGAAAATATC R: AGTGGCAAGATTGCAAACCG

2.2.5 P22 transduction

Transduction by bacteriophage P22 HT105/1 *int-201*, which was a kind gift from Professor Jay Hinton (University of Liverpool), was used to introduce mutations into *S*. Typhimurium DT193 field isolate L00168 (Table 2.4). P22 lysate stock prepared from JH3218 was used to transduce the Δ*fliC* (CM^r)[1] knockout to create GC001. Similarly, P22 lysate prepared from JH3219 was used to transduce the Δ*fljB* (KAN^r)[2] knockout into L00168 and GC001 to create GC002 and GC003, respectively.

2.2.5.1 Production of P22 transducing lysate

An overnight culture of the donor *Salmonella* isolate was grown in 10 ml LB broth. This culture was subcultured 1:100 (v/v) into 5 ml fresh medium in a 15 ml Falcon™ tube and grown at 37°C, 150 rpm to an OD_{600} of 0.15-0.20. P22 phage stock was added at 1:1000 (v/v) and incubated under the same conditions for 6 h. 100 μl chloroform was added, followed by brief shaking and static incubation at 4°C for 2 h to lyse the donor *Salmonella* cells. Cellular debris was removed from the lysed culture by centrifugation at 1,000 x *g* for 15 min and the supernatant was filtered using a 0.22 μm syringe filter unit (Millipore Ltd., UK). Lysate stock was stored in a glass universal over 100 μl chloroform at 4°C.

[1] CM^r, chloramphenicol resistance

[2] KAN^r, kanamycin resistance

2.2.5.2 Transduction with P22 lysate

The *Salmonella* isolate to be transduced was grown overnight in 10 ml LB broth at 37°C, 150 rpm for 16-18 h. 200 µl of recipient *Salmonella* was added 1:20 (v/v) to 10 µl of P22 lysate and incubated at 37°C, 150 rpm for 1 h to allow the transduced cells to express the acquired antibiotic resistance genes. To select for positive transductants, 100 µl of transduced bacteria were plated onto nutrient agar containing the appropriate selective antibiotic (20 µg/ml chloramphenicol or 50 µg/ml kanamycin). Negative controls were prepared by spreading 100 µl recipient *Salmonella* with no phage and 50 µl of P22 lysate onto separate nutrient agar plates supplemented with appropriate antibiotic. Nutrient agar plates were incubated for 20-24 h at 37°C and then inspected for growth of antibiotic resistant colonies (transductants).

2.2.5.3 Purification of transductants

Transductants were picked from single colonies and streaked onto Evans Blue Uranine (EBU) agar plates to prevent the formation of stable lysogens and differentiate pseudolysogens from phage-free non-lysogens. EBU agar plates were incubated at 37°C for 20-24 h after which light-coloured colonies were picked, streaked once more onto fresh EBU plates and incubated again at 37°C for 20-24 h. Single light-coloured colonies were then streaked onto nutrient agar with selective antibiotic and incubated under the same conditions. Mutations in purified transductants were confirmed by PCR using the primers listed in Table 2.3.

2.2.6 Transmission electron microscopy

For visualisation of *Salmonella* bacterial cells by transmission electron microscopy (TEM), stationary phase cultures were prepared by inoculating 10 ml LB broth with a few colonies from a nutrient agar plate and incubating for 16-18 h at 37°C, 150 rpm. 10 µl of bacterial suspension was placed on a Formvar/carbon-coated copper grid (300 mesh) and allowed to dry for 15-20 min at ambient temperature. Excess suspension was removed with filter paper and the grid was washed twice for 1 min with dH_2O. Samples were negatively stained with 2% (w/v) uranyl acetate (pH 4.2) for 45 s, after which the stain was drained off and the grid was allowed to dry. Stained samples were viewed at 80 kV with a Philips EM 208S TEM.

Table 2.4 Lab isolates of *Salmonella* used in this study and their antimicrobial resistance profile.

Isolate	Relevant genotype	Resistance profile	Reference
JH3218	SL1344*; Δ*fliC*	CM	Arques *et al*., 2009
JH3219	SL1344*; Δ*fljB*	KAN	Arques *et al*., 2009
GC001	L00168 DT193; Δ*fliC*	CM, T, A, S, SU	This study
GC002	L00168 DT193; Δ*fljB*	KAN, T, A, S, SU	This study
GC003	L00168 DT193; Δ*fliC* Δ*fljB*	CM, KAN, T, A, S, SU	This study
4/74 Δ*prgH*	4/74; *prgH* ::mini-Tn*5*Km2	KAN, NA	Morgan *et al*., 2004

**Salmonella* strain SL1344 derives from strain 4/74

Abbreviations: CM, chloramphenicol; KAN, kanamycin; T, tetracycline; A, ampicillin; S, streptomycin; SU, sulfamethoxazole

2.2.7 Motility assays

Two to three colonies of *Salmonella* isolates picked from nutrient agar plates were grown in 10 ml LB broth at 37°C, 150 rpm for 16-18 h before being diluted 1:100 (v/v) in fresh LB medium and grown for a further 3.5 h. Subsequently, 1 μl of these late-log cultures were spotted in the centre of semi-solid LB plates containing 0.25% agar and left to dry at room temperature for 1 h. Plates were incubated aerobically at 37°C for 6 h and haloes of growth were measured in millimetres.

2.2.8 Assessment of biofilm formation

2.2.8.1 Biofilm formation on polystyrene

For assessment of the extent of bacterial adherence to polystyrene, overnight cultures of *Salmonella* were diluted in fresh LB broth to achieve an OD_{600} of 0.2. *S.* Enteritidis PT4 isolate P125109 was used as a positive control as this serotype has been shown to form biofilms (Solano *et al*., 2002; Woodward *et al*., 2000). 30 μl of bacterial suspension was added to wells of a 96 well polystyrene microtitre plate

containing 100 µl of LB broth. The microtitre plates were incubated statically at room temperature or 37°C for 24 h, 48 h, or 1 week. After incubation, supernatants were removed by aspiration, loosely adherent bacteria were removed by washing three times with PBS and the plates were inverted to dry at room temperature. Adherent bacteria were stained by addition of 100 µl 1% (w/v) crystal violet to each well for 30 min at room temperature. Unbound crystal violet was removed by extensive washing of the microtitre plates with sterile dH_2O and the plates were inverted to dry again at room temperature. 130 µl ethanol:acetone (80:20 v/v) was added to each well to solubilise the cell-bound crystal violet. After 10 min incubation at room temperature, optical density readings at 600 nm were determined using a Multiskan FC Microplate reader (Thermo Scientific, UK).

2.2.8.2 Visualisation of biofilms

For examination of the structure of biofilms by microscopy, the *Salmonella* isolates were grown on glass chamber slides. First, overnight cultures were grown in 10 ml LB broth at 37°C, 150 rpm and diluted to an OD_{600} of 0.2 with fresh LB. Wells of a chamber slide were filled with 100 µl diluted bacterial suspension and 400 µl sterile LB broth. Slides were incubated at room temperature or 37°C for 1 week, with daily replacement of LB broth. After the incubation period, the medium was decanted from the wells and the slides were washed three times with sterile PBS to remove debris. The gasket and any remaining silicone gel was then detached from the glass slide using a scalpel and the slides were heat fixed by brief passing through a flame with forceps. The slides were flooded with crystal violet and left to stain for 3 min, after which excess stain was drained off and slides were washed extensively with PBS. Slides were visualised using a Nikon Eclipse 80i microscope with an oil-immersion (100x) lens.

2.2.9 Analysis of secreted proteins

2.2.9.1 Collection of proteins

For visual analysis of the proteins secreted into culture medium, *Salmonella* isolates were grown for 16-18 h in 10 ml LB broth supplemented with 300 mM NaCl at 37°C, 150 rpm. *S.* Typhimurium 4/74 Δ*prgH* was included for visual comparison; this isolate has a mutation that affects the structure of its T3SS-1 needle complex and therefore its ability to secrete SPI-1 effector proteins. A 2 ml aliquot was collected

from each culture and bacteria were harvested by centrifugation at 8,000 x *g* for 15 min. The supernatant was filtered through a 0.45 *µ*m pore size low protein binding syringe filter unit (PVDF; Millipore Ltd., UK). Proteins in the cell-free culture supernatant were precipitated by addition of 150 *µ*l 100% ice-cold trichloroacetic acid to 1.5 ml supernatant to achieve a final concentration of 10% (v/v) trichloroacetic acid. After incubation on ice for 1 h, proteins were recovered by centrifugation at 10,000 x *g* for 20 min at room temperature. Supernatants were then discarded and the protein pellets were washed twice with 20 *µ*l ice-cold acetone and centrifuged at 10,000 x *g* for 5 min. The protein pellets were dried and resuspended in 50 *µ*l 2x Laemmli sample buffer (Bio-Rad Laboratories Ltd., UK) containing 5% (v/v) β-mercaptoethanol. The presence of residual trichloroacetic acid occasionally caused the protein suspension to turn yellow, in which case 1-2 *µ*l 1M NaOH was added. Samples were boiled at 95°C for 5 min before being loaded onto an acrylamide gel.

2.2.9.2 SDS-PAGE

Protein suspensions were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). 25 *µ*l of each sample was loaded into wells of a 10% (w/v) acrylamide Precise Tris Glycine pre-cast gel (Thermo Fisher Scientific, UK) in a Mini-PROTEAN Tetra Cell electrophoresis tank (Bio-Rad Laboratories Ltd., UK). A pre-stained broad range standard (Bio-Rad Laboratories, Ltd., UK) was included on the gel as a marker for protein molecular weight. The tank was filled with a 1x working solution of Tris-glycine-SDS running buffer prepared by 1:10 (v/v) dilution of a 10x stock solution containing 29g Tris base, 144g glycine, 10g SDS (made up to 1 L with dH_2O). The gel was run for 80 min at 100 V, followed by staining of the protein bands in Coomassie Brilliant Blue R-250 staining solution (Bio-Rad Laboratories Ltd., UK) for 30 min at room temperature with gentle agitation. The gel was then agitated in Coomassie Brilliant Blue R-250 destaining solution (Bio-Rad Laboratories Ltd., UK) at room temperature twice for 2 h, or until the bands were clear against the background. Images of the protein gels were captured under white light using a CCD imager.

2.2.10 Statistical analysis

Statistical analyses were performed using MiniTab software, version 16. Mean values and standard deviations were calculated and isolates were compared using one-way ANOVA, unless stated otherwise. Differences were considered significant when $P<0.05$. In the event that a significant difference was found, the Tukey method of post-hoc multiple comparisons was performed.

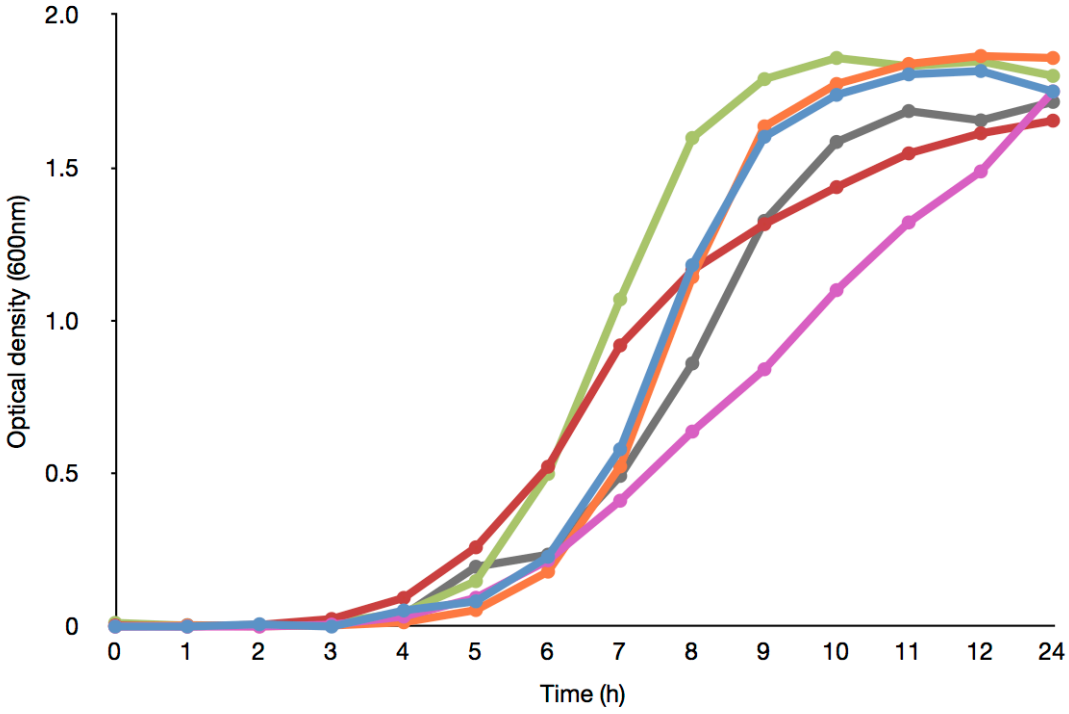
2.3 Results

2.3.1 Bacterial growth curves

To determine the growth pattern of the field isolates of *Salmonella*, bacterial cultures were monitored over 24 h for changes in OD_{600} and viable counts. This was necessary because the growth state of bacteria is known to affect virulence. For example, Lee and Falkow (1990) observed that *Salmonella* in the late-log phase of growth were more invasive into cultured cells than *Salmonella* in the early-log or stationary phase of growth.

Measurement of the optical density of the growing cultures revealed that the majority of the *Salmonella* isolates exhibited very similar patterns of growth, displaying the characteristic sigmoid curve (Figure 2.1A). The lag, logarithmic and stationary phases are easily identifiable in most cases. However, cultures of 4,5,12:i:- isolate S04327 exhibited an altered pattern of growth, with a reduced OD_{600} compared to the other isolates between 7 h and 12 h. By 24 h, the OD_{600} of this isolate had reached the same level as the others. The viable count data for S04327 also revealed slightly lower CFU/ml than the other isolates at many of the time points (Figure 2.1B).

A.



B.

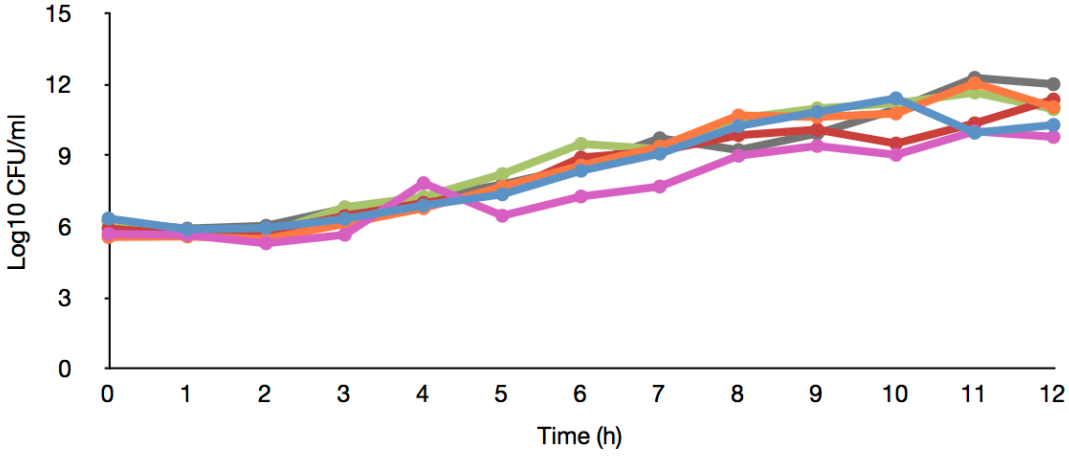


Figure 2.1 Growth of *Salmonella* cultures.

Salmonella DT193 isolates S01299 (●), S04327 (●), S03554 (●), S00398 (●), S01557 (●) and 4/74 (●) were grown in 30 ml LB broth in 75 cm^3 polystyrene flasks under microaerophilic conditions at 37°C, 150 rpm in an orbital shaking incubator. **A.** The optical density at 600 nm was measured every hour. **B.** Viable counts (log_{10} CFU/ml) were determined by serial dilution and plating onto nutrient agar. Results are means of duplicate experiments.

2.3.2 Presence of virulence-associated genes

The presence of a range of virulence-associated genes in the *Salmonella* field isolates was determined by PCR. The panel of genes used was similar to those used by others for virulotyping of *Salmonella* spp. (Dione *et al.*, 2011; Hughes *et al.*, 2008; Skyberg *et al.*, 2006). All isolates were positive for *prgH*, *sopE2*, *invA*, *sitC*, *spiC*, *sifA*, *misL*, *orfL* and *pipD* and *S.* Typhimurium isolate 4/74 was the only one that was positive for all 13 genes (Table 2.5). The *sopB* gene was present in all isolates except the *S.* Typhimurium DT193 isolate S00398 and the *iroN* gene was present in all except DT193 isolate L00168. Out of the six DT193 isolates only one (S01557) was positive for *pefA*, with all of the 4,[5],12:i:- isolates being negative for this fimbrial protein-encoding gene. Additionally, only two of the DT193 isolates were positive for *sopE*, both of which were 4,5,12:i:- isolates (S04327 and S03554). These 2 isolates were also the only pair with identical virulence gene repertoires, all of the other isolates differed by 1 or 2 virulence genes.

Table 2.5 Presence (+) and absence (-) of virulence-related genes in *Salmonella* isolates.

Isolate	Serotype	*prgH*	*sopB*	*sopE*	*sopE2*	*invA*	*sitC*	*spiC*	*sifA*	*misL*	*orfL*	*pipD*	*ironN*	*pefA*
S01299	4,12:i:-	+	+	−	+	+	+	+	+	+	+	+	+	−
S04327	4,5,12:i:-	+	+	+	+	+	+	+	+	+	+	+	+	−
S03554	4,5,12:i:-	+	+	+	+	+	+	+	+	+	+	+	+	−
S00398	Typhimurium	+	−	−	+	+	+	+	+	+	+	+	+	−
S01557	Typhimurium	+	+	−	+	+	+	+	+	+	+	+	+	+
L00168	Typhimurium	+	+	−	+	+	+	+	+	+	+	+	−	−
4/74	Typhimurium	+	+	+	+	+	+	+	+	+	+	+	+	+

2.3.3 Presence of flagella and flagella-associated genes

PCR analysis confirmed that the field isolates of *Salmonella* 4,[5],12:i:- were negative for the *fljB* gene (Table 2.6 and Figure 2.2). However, despite the absence of this gene, visualisation of surface structures by TEM revealed that the monophasic isolates possessed peritrichous flagella (Figure 2.3). The flagella of the 4,[5],12:i:- isolates were morphologically indistinguishable from those of the phase variable DT193 isolates. In addition to being negative for the *fljB* gene, all three of the 4,[5],12:i:- field isolates were negative for *fljA* and *hin*. The transmission electron micrographs (Figure 2.3) confirm that the 4,[5],12:i:- isolates are able to construct full-length flagella.

The PCR results also confirmed that P22 transduction of L00168 to produce the knockout mutants GC001, GC002 and GC003 was performed correctly; the desired genes were knocked out, giving appropriate negative results. Examination of the Δ*fljB* mutant (GC002) by TEM revealed the presence of truncated flagella only (Figure 2.3E). This isolate seemed unable to construct full-length flagella filaments. A possible explanation is that although the *fljB* gene was knocked-out, the *fliC* repressor gene, *fljA*, was still present and could still be expressed, resulting in reduced expression of FliC flagella protein.

In addition to flagella, examination of TEM images revealed the presence of fimbriae on some of the isolates (Figure 2.4). Fimbriae are hair-like appendages found on the surface of *Salmonella* cells that mediate attachment to epithelial cells during the intestinal stage of infection. They were found on both a monophasic isolate (S01299) and a flagellar phase variable isolate (L00168).

Table 2.6 Presence (+) and absence (-) of flagella-associated genes in *Salmonella* isolates.

Isolate	Serotype	*fliC*	*fljB*	*fljA*	*hin*
S01299	4,12:i:-	+	–	–	–
S04327	4,5,12:i:-	+	–	–	–
S03554	4,5,12:i:-	+	–	–	–
S00398	Typhimurium	+	+	+	+
S01557	Typhimurium	+	+	+	+
L00168	Typhimurium	+	+	+	+
GC001 (Δ*fliC*)	Typhimurium	–	+	+	+
GC002 (Δ*fljB*)	Typhimurium	+	–	+	+
GC003 (Δ*fliC* Δ*fljB*)	Typhimurium	–	–	+	+
4/74	Typhimurium	+	+	+	+

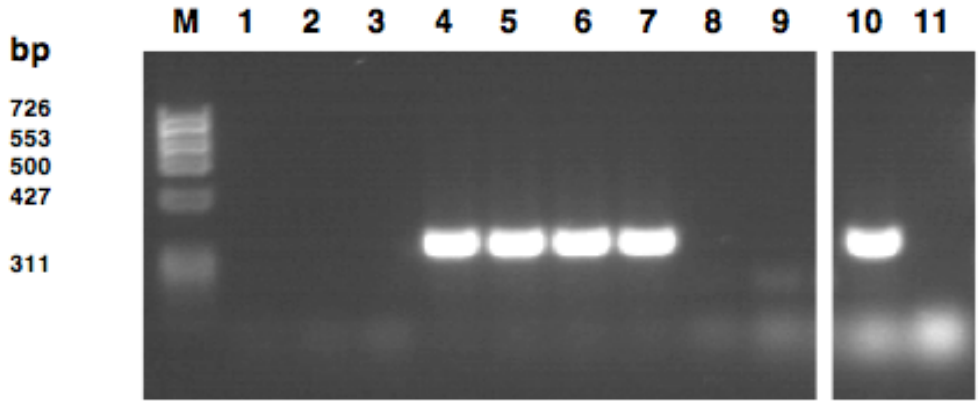


Figure 2.2 PCR analysis of presence of *fljB* gene.

Gel image of products from PCR reactions containing the primer for *fljB* and DNA of *Salmonella* DT193 isolates. The lane marked 'M' contained ΦX174 HaeIII digest DNA marker, whose product molecular weights (in base pairs, bp) are indicated on the left-hand side. Lane 1, S01299; lane 2, S04327; lane 3, S03554; lane 4, S00398; lane 5, S01557; lane 6, L00168; lane 7, GC001 (Δ*fliC*); lane 8, GC002 (Δ*fljB*); lane 9, GC003 (Δ*fliC* Δ*fljB*); lane 10, ST 4/74; lane 11, negative control.

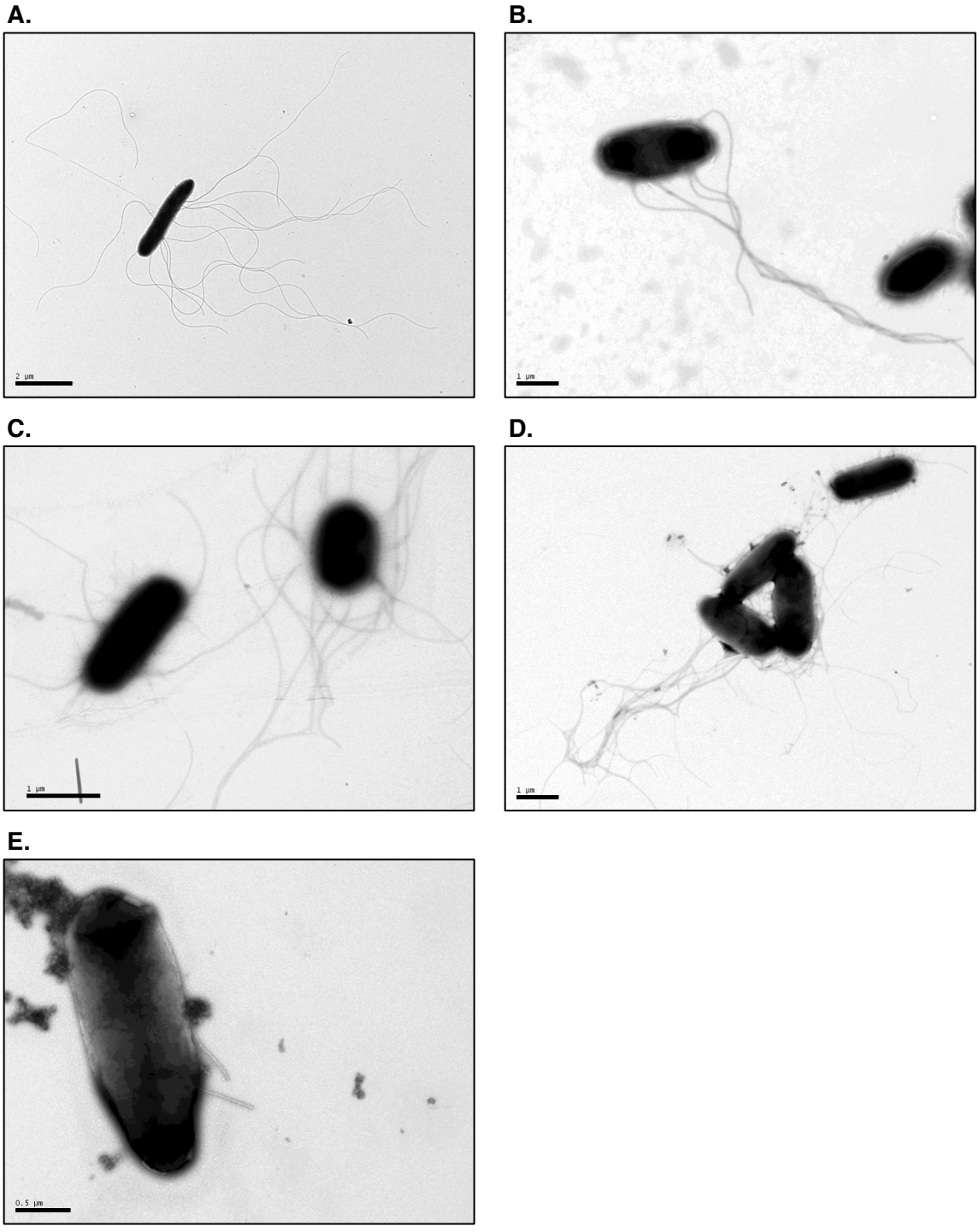


Figure 2.3 Peritrichous flagella of *Salmonella* DT193 isolates.

Transmission electron micrographs showing the presence of flagella on *Salmonella* 4,[5],12:i:- DT193 isolates S01299 (**A**) and S04327 (**B**); *S*. Typhimurium DT193 isolates S01557 (**C**) and L00168 (**D**); Δ*fljB* knockout of L00168 (**E**). The flagella present on the Δ*fljB* mutant (**E**) appear to be stunted in length compared to the parent strain L00168 (**D**). Negative staining and image capture was performed by Miss Marion Pope (Veterinary Pathology, University of Liverpool).

A. **B.**

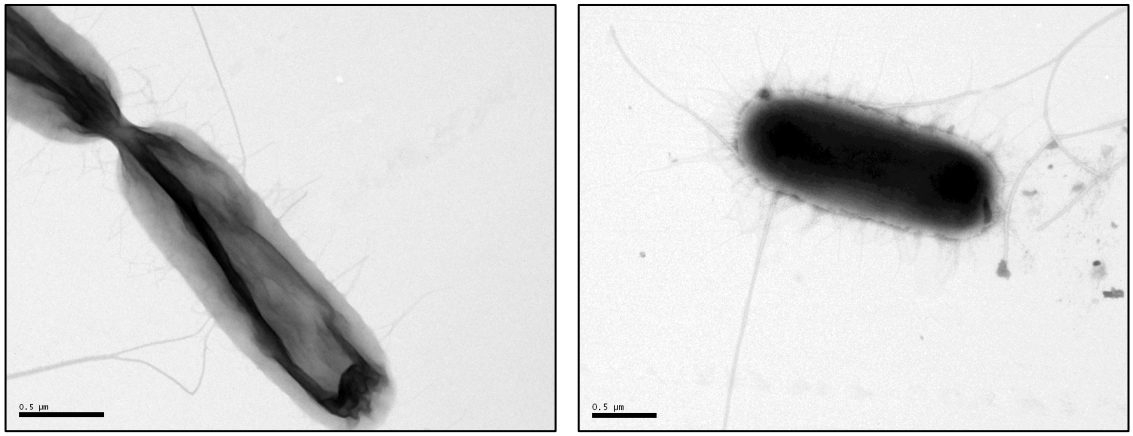


Figure 2.4 Fimbriae of *Salmonella* DT193 isolates.

Transmission electron micrographs showing the presence of fimbriae on *Salmonella* 4,12:i:- isolate S01299 (**A**) and *S.* Typhimurium isolate L00168 (**B**). Negative staining and image capture was performed by Miss Marion Pope (Veterinary Pathology, University of Liverpool).

2.3.4 Motility

Given that 4,[5],12:i:- isolates do not express the second flagellar phase protein FljB, it was necessary to determine whether this has any effect on motility. The *Salmonella* isolates were inoculated into the centre of semi-solid agar plates, from which point the bacterial cells collectively migrated radially outwards along a chemotaxis gradient after depletion of local nutrients. This type of motility, through an aqueous environment, is known as "swimming" and is different to the type of motility employed by flagellated bacteria for migration across a solid surface, which is known as "swarming". Measurement of the halo of migration visible on the semi-solid agar plates served as an assessment of each isolate's motility (Figure 2.5). Results of the motility assays (Figure 2.6) revealed that the flagellar phase variable isolate S01557 was significantly less motile than *S.* Typhimurium 4/74 and all of the other field isolates (P = 0.0000). All three of the 4,[5],12:i:- isolates exhibited a similar level of motility to the remaining biphasic isolates (S00398 and L00168) and to 4/74.

A. **B.**

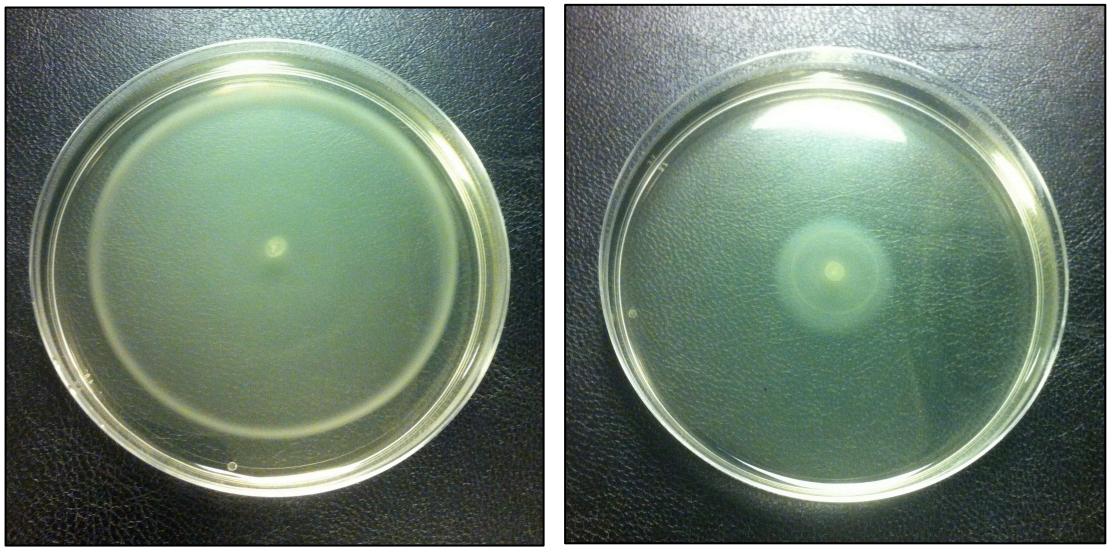


Figure 2.5 Haloes of growth of *Salmonella* DT193 isolates on swim plates.

Representative images of motility assays of *Salmonella* 4,5,12:i:- isolate S03554 (**A**) and *S.* Typhimurium isolate S01557 (**B**). 1 *µ*l of late-log phase bacterial culture was spotted in the centre of a semi-solid LB plate containing 0.25% (w/v) agar. After 6 h incubation at 37°C the diameter of the halo of migration around the central point of inoculation was measured. Isolate S01557 exhibited reduced motility, resulting in a smaller halo (**B**).

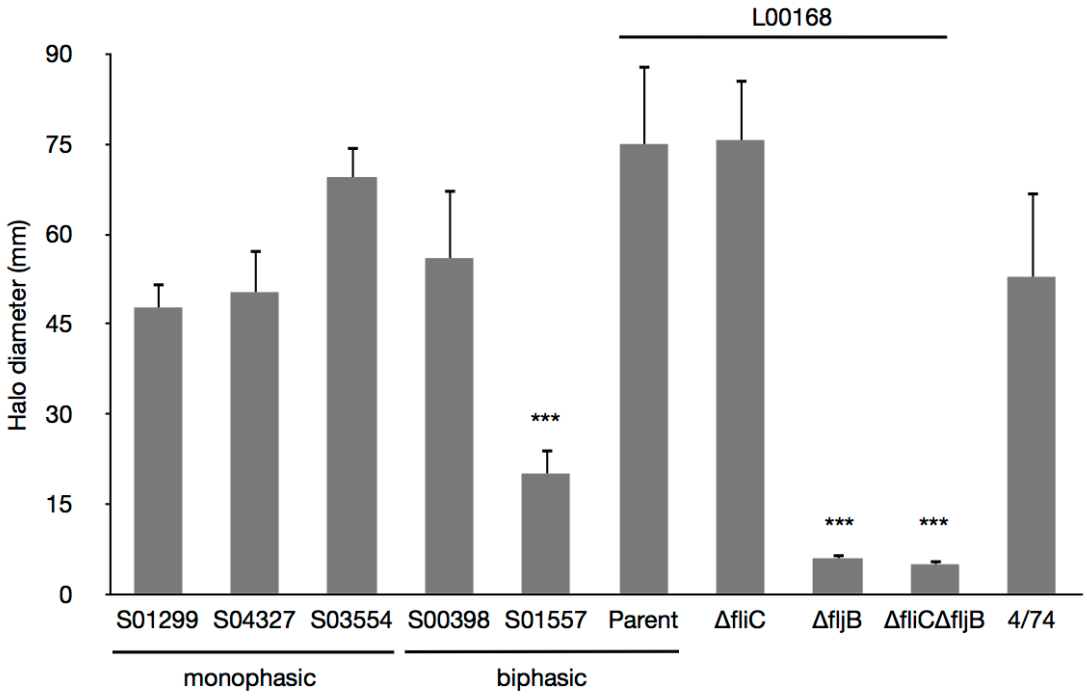


Figure 2.6 Comparison of motility of *Salmonella* isolates.

Swimming motility of monophasic (4,[5],12:i:-) and biphasic DT193 isolates was assessed by spotting 1 μl of late-log phase cultures in the centre of semi-solid LB plates containing 25% (w/v) agar. After 6 h incubation at 37°C the diameter of the halo of migration around the central point of inoculation was measured. The motility of isogenic ΔfliC (GC001), ΔfljB (GC002) and ΔfliC ΔfljB (GC003) mutants of L00168 was also assessed. *S*. Typhimurium 4/74 was included as a positive control. Results are mean ± SD of triplicate experiments, where n = 3. Isolates with statistically significantly lower mean motility, compared to the other isolates, are indicated with asterisks, where P = 0.0000, determined by one-way ANOVA with Tukey post-hoc comparison.

2.3.5 Biofilm formation

Biofilms are sessile communities of microorganisms that grow on surfaces embedded within a matrix of extracellular material. Bacteria within biofilms are protected against the external environment, conferring resistance to disinfection and drying ((Møretrø *et al.*, 2009; Scher *et al.*, 2005; White *et al.*, 2006). To determine the degree of biofilm formation on polystyrene by the *Salmonella* isolates after 24 h, 48 h or 1 week incubation, adherent bacteria and biofilm matrix components were stained with crystal violet. Biofilm formation was evaluated at both 37°C and room temperature (~25°C) to represent temperature conditions in a host and in the environment, respectively.

Figure 2.7 provides a visual representation of the overall main effects of time and temperature on biofilm formation. A main effect is the effect of a variable (e.g. time) averaged over levels of the other variable (e.g. temperature). The main effects plot indicates that on average, across all of the isolates, the amount of biofilm increased with time and slightly more biofilm was formed during incubation at room temperature (~25°C) than at 37°C. Statistical analysis of the main effects, using a repeated measures ANOVA, revealed that the OD_{600} values were significantly different at the different time points ($P = 0.000$) and at the different temperatures ($P = 0.022$). This analysis also revealed that the interaction between time and temperature was significant ($P = 0.000$). An interaction occurs when the effect of a variable depends on the level of another variable. A Tukey post-hoc comparison test identified the primary source of the interaction; the mean OD_{600} after 1 week of incubation was significantly higher at room temperature than at 37°C ($P = 0.0001$).

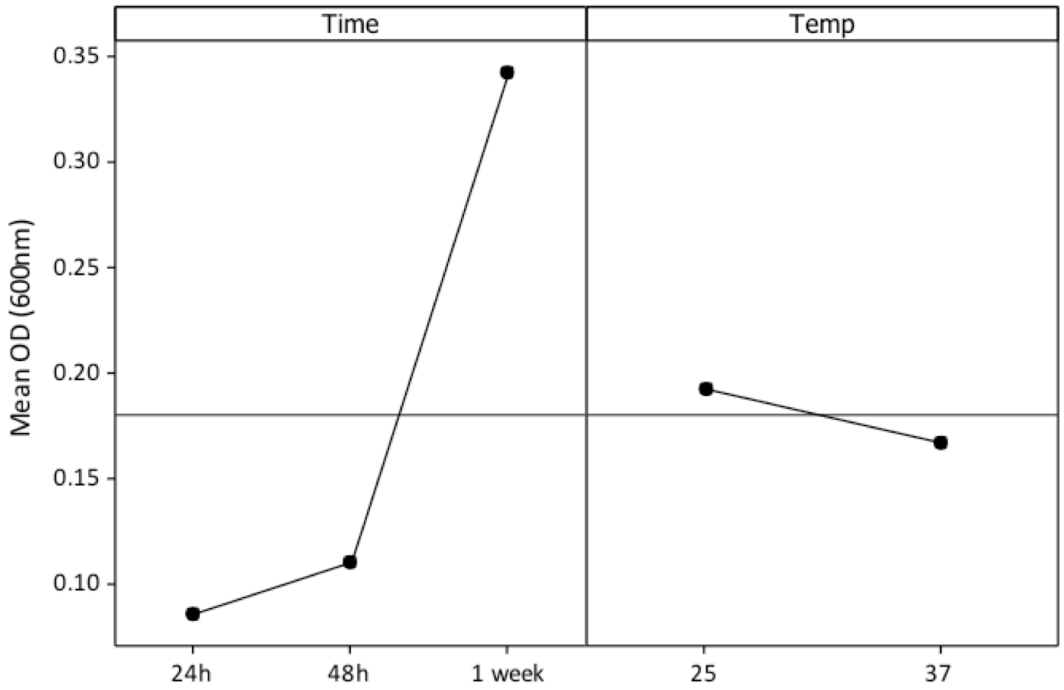


Figure 2.7 Main effects plot of time and temperature on biofilm formation by *Salmonella* isolates on polystyrene.

The ability of *Salmonella* isolates to form a biofilm on polystyrene was assessed by staining of adherent bacteria and biofilm matrix components in 96 well microtitre plates. Cultures of *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates, plus *S.* Typhimurium 4/74 and *S.* Enteritidis P125109 were inoculated into wells of a microtitre plate and incubated at room temperature (25°C) or 37°C for 24 h, 48 h or 1 week. Loosely adherent bacteria were removed by washing with PBS and adherent bacteria were stained with crystal violet. Level of staining was determined by measurement of OD at 600 nm. Results presented are the overall main effects of temperature and time (independent variables) on OD_{600} (dependent variable). Data are the combined mean for all isolates from triplicate experiments, where n = 3.

2.3.5.1 Biofilm formation after 24 hours

The ability of each individual isolate to form a biofilm on polystyrene at the three levels of time and two levels of temperature is presented in

Figure 2.8. Data from each time point were treated separately and two-way analyses of variance with Tukey post-hoc comparisons were used to identify the effect of temperature and *Salmonella* strain on biofilm formation. At 24 h (

Figure 2.8A), there was a significant difference between the biofilms produced by the different isolates (P = 0.000) and this was dependant on the temperature at which the isolates were incubated (P = 0.000). Specifically, the biofilm of 4,12:i:- isolate S01299 produced a significantly higher OD_{600} at 37°C than at room temperature (P = 0.0001), as did the biofilm of *S*. Enteritidis P125109 (P = 0.01). Both S01299 and *S.* Enteritidis P125109 formed a greater amount of biofilm than all of the other isolates when incubated at 37°C ($P < 0.01$). There was no significant difference between the mean OD_{600} of S01299 and *S*. Enteritidis P125109 when grown at either temperature. The mean OD_{600} of the isolates ranged from 0.06-0.12 after 24 h growth, which is low compared to the optical densities of *S*. Typhimurium isolates grown under similar conditions in another study (Vestby *et al.*, 2009).

2.3.5.2 Biofilm formation after 48 hours

Results after 48 h incubation (

Figure 2.8B) were very similar to those after 24 h incubation. However, by this time point there was a significant difference between the amount of biofilm created by S01299 and *S*. Enteritidis P125109 when incubated at room temperature (P = 0.0001), whereas at 24 h there was not. The OD_{600} of the *S*. Enteritidis biofilm produced at room temperature was now similar to that produced at 37°C. Growth of S01299 at 37°C and *S*. Enteritidis at both temperatures resulted in formation of significantly greater biofilm than all other isolates ($P<0.05$).

2.3.5.3 Biofilm formation after 1 week

After incubation for 1 week (

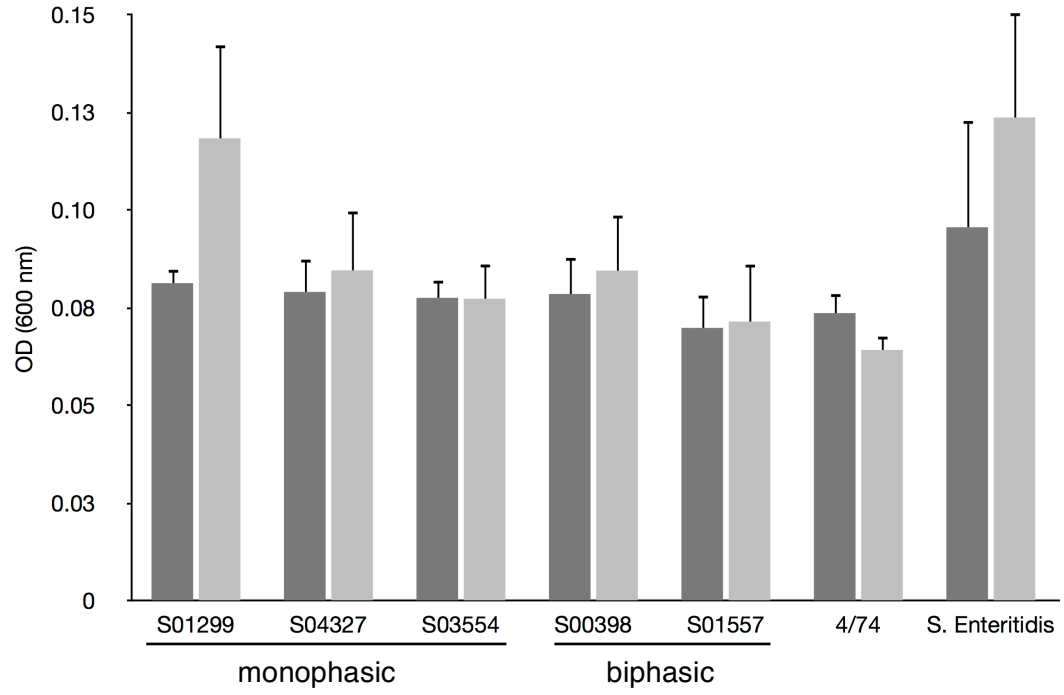
Figure 2.8C), there were no significant differences between the amounts of biofilm formed by the different isolates (P = 0.208), although this was probably due to large variation in results at this time point. However, incubation at the two different

temperatures did result in significantly different amounts of biofilm formed (P = 0.003). Specifically, the biofilm of 4,5,12:i:- isolate S03554 produced a significantly higher OD_{600} when incubated at room temperature compared to at 37°C (P = 0.017). *S.* Typhimurium isolates S01557 and 4/74 also produced more biofilm when incubated at room temperature than when grown at 37°C, but the differences were not statistically significant. The increase in optical density of the biofilm produced at room temperature by S03554, S01557 and 4/74 between 48 h and 1 week was quite large, from 0.11 to 0.48, 0.08 to 0.47 and 0.09 to 0.51, respectively. Whereas the increase in optical density between these time points at room temperature for *S*. Enteritidis was only marginal, from 0.22 to 0.24.

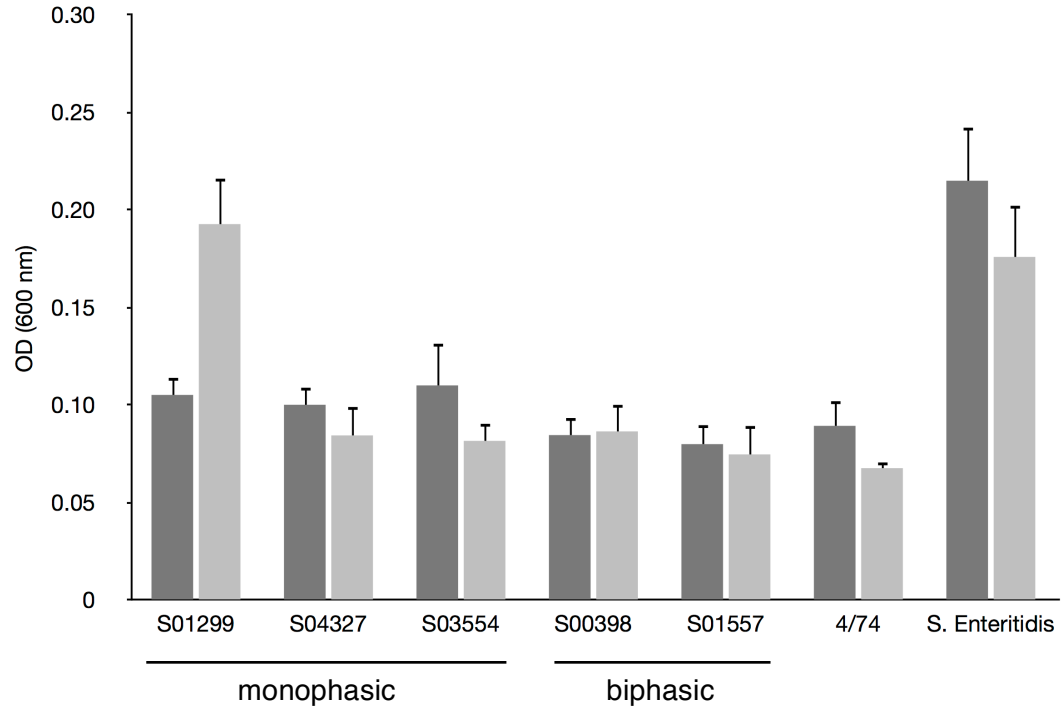
2.3.5.4 Biofilm formation on glass

The ability of the *Salmonella* isolates to form biofilms on glass was also qualitatively assessed by microscopy (Figure 2.9). Because the optical densities of the biofilms formed on polystyrene were so low at 24 h and 48 h, biofilms were only microscopically examined after incubation for 1 week. On glass, isolate S01299 produced more of a biofilm at 37°C than at room temperature. Isolates S03554 and 4/74 on the other hand produced more of a biofilm at room temperature than at 37°C. *S*. Enteritidis P125109 showed some evidence of biofilm formation on glass, but not to the same extent as the *S*. Typhimurium isolates. The results for biofilm formation on glass were comparable to those observed on polystyrene after 1 week. Another study that compared the adherence of *S.* Typhimurium to glass and polystyrene surfaces also showed that cell distribution was comparable on both (Römling & Rohde, 1999).

A.


0.15
0.13
0.10
0.08
0.05
0.03
0
OD (600 nm)
S01299
S04327
S03554
S00398
S01557
4/74
S. Enteritidis
monophasic
biphasic

B.


0.30
0.25
0.20
0.15
0.10
0.05
0
OD (600 nm)
S01299
S04327
S03554
S00398
S01557
4/74
S. Enteritidis
monophasic
biphasic

C.

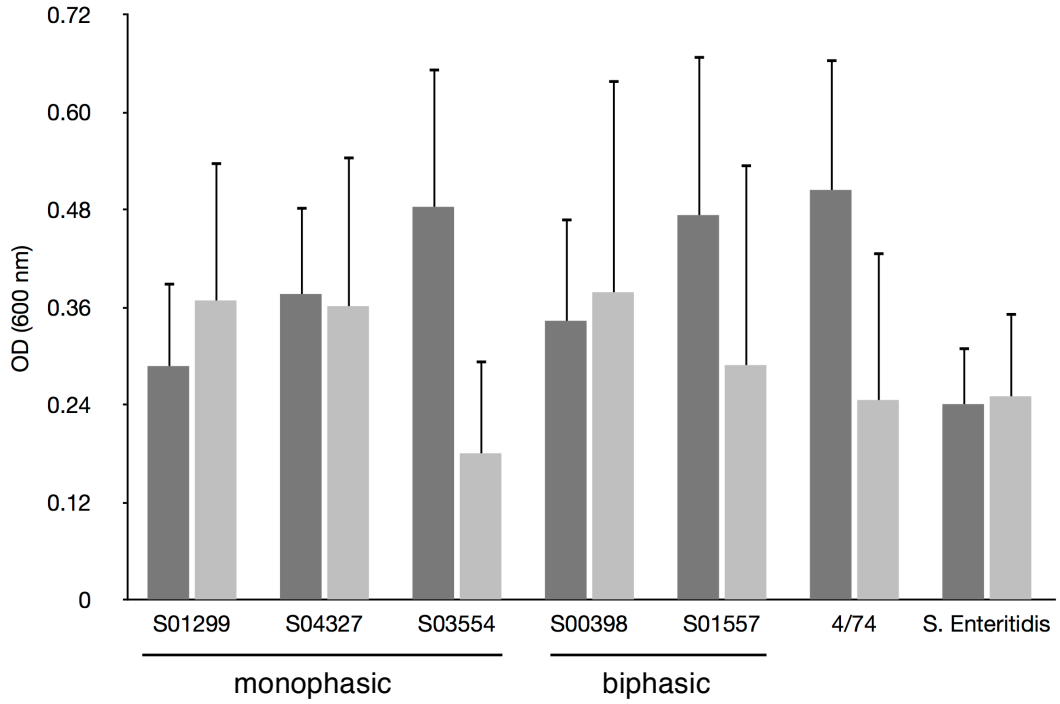


Figure 2.8 Biofilm formation by *Salmonella* isolates on polystyrene.

Cultures of *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates, plus *S.* Typhimurium 4/74 and *S*. Enteritidis P125109 were inoculated into wells of a microtitre plate and incubated at room temperature (■) or 37°C (■) for 24 h (**A**), 48 h (**B**) or 1 week (**C**). Adherent bacteria were stained with crystal violet and the level of staining was determined by measurement of OD at 600 nm. Data are mean values ± SD of triplicate experiments, where n = 3.

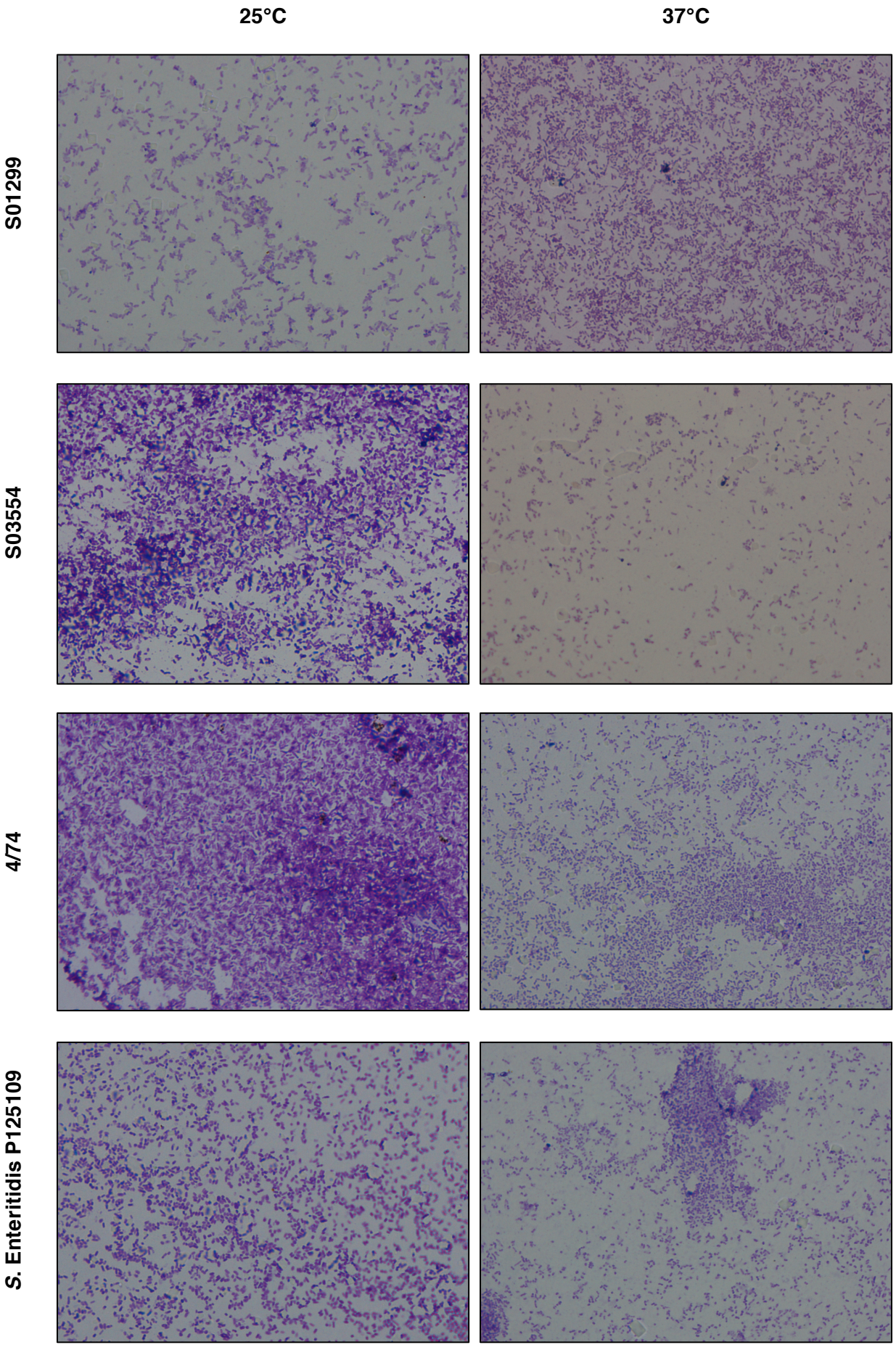

25°C
37°C
S01299
S03554
4/74
S. Enteritidis P125109

Figure 2.9 Biofilm formation by *Salmonella* isolates on glass.

Representative microscopy images of *Salmonella* cell distribution on a glass surface. Cultures of *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates, plus *S.* Typhimurium 4/74 and *S.* Enteritidis P125109 were inoculated into wells of a chamber slide with LB broth. Slides were incubated at room temperature (~25°C) or 37°C for 1 week. Adherent cells and biofilm extracellular matrix were stained with crystal violet and slides were viewed using a Nikon Eclipse 80i microscope with an oil-immersion lens. Magnification, x1000.

2.3.6 Secretion of proteins into culture media

Culturing *Salmonella* in high osmolarity, a condition resembling that found within the host intestinal lumen, stimulates the expression of components and substrates of the SPI-1 T3SS (Chen *et al.*, 1996b; Galán & Curtiss III, 1990; Møretrø *et al.*, 2009). These substrates, otherwise known as SPI-1 effector proteins, are usually exported into the cytosol of host cells during invasion, via a T3SS. However, in the absence of a host cell, some of these proteins are emitted into the bacterial culture media (Komoriya *et al.*, 1999). Flagellar biogenesis also involves exportation of proteins using a T3SS and sometimes these flagellar proteins are accidentally secreted into the culture media as well. Precipitating the proteins out of the culture media and running them on an SDS-PAGE gel enables qualitative assessment of the profile of secreted proteins.

Figure 2.10 shows a representative image of the pattern of secreted proteins from the different *Salmonella* isolates. The molecular weights of the secreted proteins were estimated from the broad range ladder and their identities were subsequently predicted. The most obvious bands visible on the gels appeared to have very similar molecular weights to those identified by Komoriya *et al.* (1999). In the aforementioned study, the authors applied blotted secreted proteins to an amino acid sequencer and identified an 89 kDa protein as SipA, a 67 kDa protein as SipB, a 52 kDa protein as FliC, a 50 kDa protein as FliD and a 42 kDa protein as SipC. An additional band shown in Figure 2.10 was predicted to be SipD, the molecular weight of which is 37 kDA (UniProtKB database). The Sip proteins are secreted by the SPI-1 T3SS and all except SipA are essential for bacterial entry into host cells (Akasaki *et al.*, 2006; Kaniga *et al.*, 1995a; Kaniga *et al.*, 1995b; Scherer *et al.*, 2000). *S.* Typhimurium 4/74 Δ*prgH* was negative for all of the suspected Sip protein

bands, which supports their predicted identity. The bands predicted to be SipA, SipB and SipD all appeared to be less intense in the lanes containing samples from the three 4,[5],12:i:- DT193 isolates, suggesting that these proteins were secreted in lower amounts from these isolates.

The bands predicted to be flagellar proteins were very strong, suggesting that they were secreted in large amounts into the culture media. The presence of a protein with an approximate molecular weight of 52 kDa in the lanes of the three biphasic *S*. Typhimurium DT193 isolates, that was absent from the lanes of the three 4,[5],12:i:- isolates, lead to its identity being predicted as FljB, the second phase flagellin protein. However, this protein was also absent from the lanes of GC001 (Δ*fliC*), ST 4/74 and ST 4/74 Δ*prgH*, all of which should be positive for FljB. It could be that the second of the two flagellar protein bands on the gel is a mixture of FljB, FliC and FliD, all of which have very similar molecular weights and the upper band is a different protein altogether. Therefore, without sequence analysis of the proteins present in the SDS gel, the results presented here are inconclusive. However, in general, the patterns of proteins secreted from the 4,[5],12:i:- DT193 isolates were very similar to those of the biphasic *S*. Typhimurium DT193 isolates.

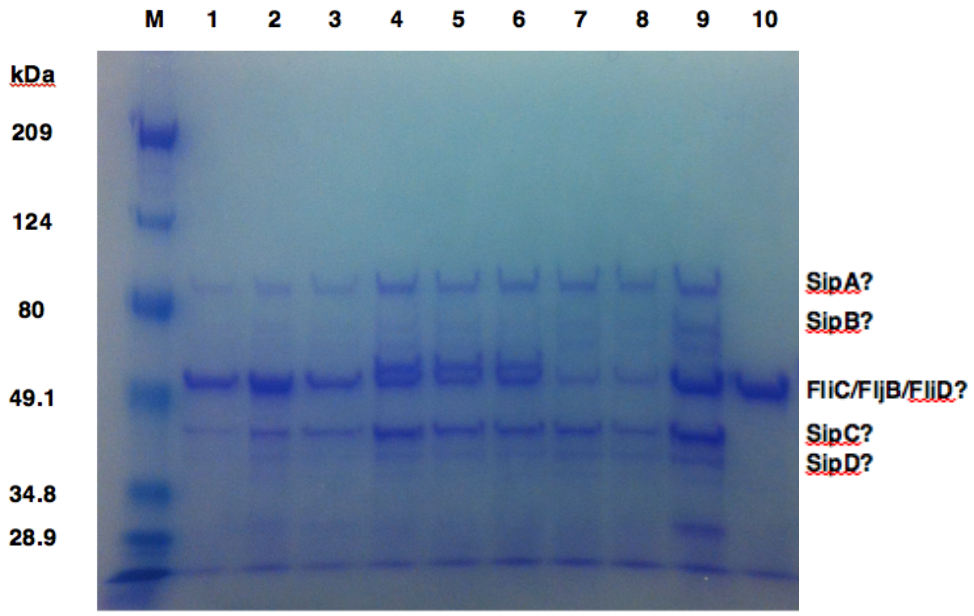


Figure 2.10 SDS-PAGE gel patterns of secreted proteins from *Salmonella* isolates.

Gel image of proteins secreted into bacterial culture media (LB broth supplemented with 300 mM NaCl) by *Salmonella* isolates. Proteins were precipitated with trichloroacetic acid and 25 μl of each sample was run on a 10% (w/v) acrylamide gel at 100V for 80 min. Protein bands were stained with Coomassie Brilliant Blue staining solution. The lane marked 'M' contained a pre-stained broad range standard, whose product molecular weights (in kDa) are indicated on the left-hand side. Lane 1, S01299; lane 2, S04327; lane 3, S03554; lane 4, S00398; lane 5, S01557; lane 6, L00168; lane 7, GC001 (Δ*fliC*); lane 8, GC002 (Δ*fljB*); lane 9, 4/74; lane 10, 4/74 Δ*prgH*. Suspected proteins represented by some of the bands are indicated on the right hand side.

2.4 Discussion

Pig-associated *Salmonella* isolates represent a significant threat to human health through consumption of contaminated pork (Pires & Hald, 2010; Pires *et al.*, 2010). A panel of monophasic *Salmonella* (4,[5],12:i-) and *S.* Typhimurium phage type DT193 field isolates originating from British pig farms were selected for phenotypic analysis because, beyond antibiotic resistance profiles, current knowledge of the phenotypic traits of isolates belonging to this emerging epidemic strain is very limited. Also, it is not currently known whether the lack of expression of the second phase flagellar antigen has any effect on the fitness of monophasic *Salmonella*. Determining the phenotype of these isolates is an important step towards understanding their virulence potential and epidemiological success.

It was necessary to first determine the growth characteristics of the isolates because growth is a fundamental indicator of bacterial fitness. Observation of bacterial growth curves revealed that growth of the 4,[5],12:i:- DT193 isolates was not noticeably different to that of the biphasic Typhimurium DT193 isolates. This suggests that loss of *fljB* expression does not confer a beneficial or deleterious effect on fitness in terms of growth. It has been postulated that loss of certain genes by genomic deletion can result in faster bacterial growth rate due to reallocation of energy expenditure from protein production to fitness-improving processes (Koskiniemi *et al.*, 2012). For example, use of flagella is an energy-hungry activity, costing a bacterial cell approximately 4.5% of its total energy expenditure (Koskiniemi *et al.*, 2012; Mitchell, 1991). Therefore, it is understandable that Koskiniemi *et al.* (2012) observed an increased growth rate in a non-motile *S.* Typhimurium Δ*fliG* mutant. Presumably, although the monophasic 4,[5],12:i:- isolates have lost *fljB*, this does not result in energy savings because they still express flagella filament via the *fliC* gene.

Two of the isolates, *S.* 4,5,12:i:- S04327 and *S.* Typhimurium S00398 exhibited growth patterns that deviated from the classic sigmoidal growth curve. Interestingly, S04327 and S00398 both have the widest repertoires of antimicrobial resistance among the panel of isolates, with resistance to ten and eleven antimicrobials, respectively (Table 2.1). Mutations that confer antimicrobial resistance often lead to a loss of fitness reflected, for example, in a reduced growth rate *in vivo* and *in vitro* (reviewed by Andersson & Hughes, 2010 and Zhang *et al.*, 2006). It is possible that

these two isolates have altered metabolism due to the antimicrobial resistance they have acquired.

In addition to acquisition of resistance to antimicrobials, expression of certain virulence phenotypes has been shown to impose a growth penalty on *Salmonella* cells (Sturm *et al.*, 2011). One such virulence phenotype is the expression of the SPI-1 T3SS needle complex, which is responsible for translocating a series of effector proteins into the cytosol of host cells. These proteins trigger rearrangements in the host cell actin and subsequent engulfment of the bacterium (Finlay *et al.*, 1991; Hardt *et al.*, 1998a). Because of the involvement of the SPI-1 T3SS and its associated effector proteins in virulence, the presence of some of the SPI-1 genes in the genomes of the panel of isolates was determined. The presence of additional virulence-associated genes encoded by other *Salmonella* Pathogenicity Islands (-2, -3, -4 and -5) and involved in iron acquisition was also determined, to give a general assessment of virulence potential.

All of the isolates were shown to possess the SPI-1 encoded gene *prgH*, whose protein is a major constituent of the base of the SPI-1 T3SS needle complex (Kubori *et al.*, 2000). This gene is essential for invasion of epithelial cells, evidenced by *prgH* mutant bacteria being defective in epithelial endocytosis in the murine model (Behlau & Miller, 1993) and in secretion of effector proteins (Jones & Falkow, 1994; Klein *et al.*, 2000; Pegues *et al.*, 1995). One such secreted effector protein is SopB, which although secreted via the SPI-1 T3SS, is encoded within SPI-5 (Wood *et al.*, 1998). This protein is an inositol phosphatase that, in conjunction with SipA, SopE and SopE2, mediates actin cytoskeleton rearrangements through activation of Rho guanosine triphosphatases (GTPases) to facilitate bacterial entry into the host cell (Hardt *et al.*, 1998a; Norris *et al.*, 1998a; Patel & Galán, 2006; Zhou *et al.*, 2001). Other examples of SopB function include induction of phenotypic transdifferentiation of epithelial cells into M cells to promote colonisation of the gut (Tahoun *et al.*, 2012), disruption of the intestinal epithelial barrier through modulation of tight junctions (Boyle *et al.*, 2006) and establishment of the intracellular niche through manipulation of vesicular trafficking (Hernandez *et al.*, 2004).

PCR virulotyping revealed that all of the isolates possessed *sopB,* except *S.* Typhimurium S00398. Virulotyping of *S.* Typhimurium isolated from piglets with diarrhoea found that 2 out of 42 isolates were negative for *sopB* (Hur et al., 2011),

suggesting that the loss of *sopB* in S00398 is not a unique event. However, a classic study investigating the prevalence and polymorphism of SPI-1 effector proteins in a variety of *S. enterica* serotypes, phage types and genotypes found that the *sopB* gene was present in all strains, along with *sopD*, *sopE2*, *sipA*, *sipB* and *sipC* (Prager et al., 2000). Mirold *et al*., (2001) also found that *sopB* was conserved across all *Salmonella* lineages. Although, Prager *et al*. (2000) found several polymorphisms in the chromosomal loci encoding these conserved effector proteins, with the *sopB* locus demonstrating the highest degree of heterogeneity. The high number of polymorphisms in *sopB* loci may indicate a lack of selective pressure to conserve this gene. Indeed, the absence of *sopB* in S00398 may represent the final result of this: complete gene loss. *S*. Typhimurium mutants deficient in any one of SopB, SopE or SopE2 have been shown to enter host cells with high efficiency, but a mutant lacking all three effectors and a Δ*sopB* Δ*sopE* double mutant were both completely defective for entry (Zhou & Galan, 2001), demonstrating that SopB and SopE are functionally redundant in the facilitation of bacterial entry. That both *sopB* and *sopE* were absent from S00398 suggests that this isolate would be severely attenuated in invasion of host cells. Although, there is a possibility that the *sopB* gene is present in S00398, but that its nucleotide sequence has changed such that the primer used did not amplify it.

SopB is also thought to be involved in initiation of inflammatory responses in the gut. For example, *S*. Dublin *sopB* mutants had a decreased ability to induce fluid secretion and neutrophil influx in infected calf intestine loops (Norris *et al*., 1998a). However, in orally infected calves mutation of the *S*. Typhimurium *sopB* gene did not affect the severity of inflammation and diarrhoea (Tsolis *et al*., 1999a). Similarly, in a murine model of colitis, SipA, SopE and SopE2 were shown to induce intestinal inflammation independent of each other and in the absence of SopB, a finding which further demonstrates the functional redundancy of these SPI-1 effector proteins and suggests that *sopB* does not play a role in the murine model of infection (Hapfelmeier *et al*., 2004). It would be interesting to determine the severity of inflammation in pigs orally infected with S00398, a naturally occurring *sopB* deficient strain, to further investigate the role of SopB in inflammation and virulence.

The heterogeneous presence of *sopE* in the panel of isolates, with only 3 out of 7 giving a positive PCR result for this gene, is far less surprising than the absence of *sopB* in S00398. This gene is found in only some *Salmonella* serotypes and the

majority of *sopE*-positive *S*. Typhimurium strains have been associated with epidemics (Hopkins & Threlfall, 2004; Mirold *et al*., 2001; Prager *et al*., 2000). The presence of *sopE* in two out of three of the 4,[5],12:i:- DT193 isolates in this study is consistent with this association. The sequence of the *sopE* gene and its location within the chromosome of *sopE*-positive strains gives clues as to its evolutionary history. It is encoded by a cryptic bacteriophage, SopEΦ, integrated at centisome 60 of the chromosome (Hardt *et al*., 1998b; Mirold *et al*., 1999) and has no known homologs in any other Gram-negative bacteria, except for *sopE2* which is ubiquitous among *Salmonella* spp. It has therefore been proposed that *sopE* was derived from *sopE2* by gene duplication (Bakshi *et al*., 2000) and its presence in only a few *Salmonella* lineages suggests that the duplication and consequent phage-mediated transfer occurred after their divergence (Mirold *et al*., 2001). Horizontal genetic transfer of *sopE* and other virulence genes may contribute to the adaptation of *Salmonella* to new hosts and the emergence of new epidemic strains.

Following translocation into the host cell cytosol, SopE induces membrane ruffling, actin cytoskeleton rearrangements and nuclear responses by acting as a guanine nucleotide exchange factor (GEF) to activate Rho GTPases CDC42 and RAC1 (Friebel *et al*., 2001; Hardt *et al*., 1998a). It has recently been shown that activation of CDC42 and RAC1 by SopE is a pattern of pathogenesis recognised by the host because it triggers the NOD1 signalling pathway, resulting in induction of NF-κB-dependent inflammatory responses (Keestra *et al*., 2013). An implication of host cells being able to "sense" the pathogen in this way and consequently generate pro-inflammatory signals has been described by Lopez and colleagues (2012). These authors found that, in a streptomycin murine model of infection, *S*. Typhimurium SL1344, which carries the SopEΦ prophage, caused a SopE-mediated increase in inducible nitric oxide synthase (iNOS). SopE-dependent iNOS expression generated host-derived nitrate, which in turn boosted the luminal growth of *S*. Typhimurium in the inflamed intestine through nitrate respiration. Thus, possession of *sopE* gives *Salmonella* a growth advantage by causing inflammation in the gut and enhancing the production of host-derived nitrate.

Further evidence for SopE having a direct role in enteropathogenicity is that *S.* Typhimurium expression and translocation of *sopE* alone, in the absence of *sipA*, *sopB* and *sopE2*, is sufficient for invasion of tissue culture cells and induction of NF-κB (Hardt *et al*., 1998a; Mirold *et al*., 2001). Similarly, phage-mediated horizontal

transfer of *sopE* was shown to increase the enteropathogenicity of *S*. Typhimurium in a bovine ligated loop model (Zhang *et al*., 2002). While deletion of *sopE* from *S.* Typhimurium SL1344 has been reported to reduce its invasiveness (Hardt *et al*., 1998a; Mirold *et al*., 2001; Stender *et al*., 2000; Zhou *et al*., 2001), a study comparing the invasion of *S.* Typhimurium *sopE*-positive, *sopE*-negative and *sopE* deletion mutants found no consistent relationship between presence of *sopE* and invasiveness into cultured cells (Clark *et al*., 2011). However, the authors did reveal that absence of *sopE* was closely correlated with reduced size of membrane ruffles and reduced speed at which rearrangements in plasma membrane architecture were induced. The finding that two out of three 4,[5],12:i:- DT193 isolates in this study, but none of the biphasic *S*. Typhimurium DT193 isolates, possessed *sopE* is interesting and the differential expression might result in divergent pathogenicity profiles during infection. The presence of *sopE* in *S*. Typhimurium 4/74 could explain its ability to cause diarrhoea in calves (Rankin & Taylor, 1966). Investigation into the interaction of these isolates with cultured cells is explored and discussed in relation to *sopE* in Chapter 3. A literature search using the terms "sopE", "monophasic" and "*Salmonella*" returned no results, suggesting that the presence of this gene in *Salmonella* 4,[5],12:i:- has not yet been investigated. As only three monophasic isolates were included on this panel, it would be pertinent to look for *sopE* in a wide range of other *Salmonella* 4,[5],12:i:- isolates.

All of the isolates in this study were positive by PCR for *sopE2*, a gene that is expressed by all *Salmonella* strains and the protein of which is 69% identical to SopE (Bakshi *et al*., 2000; Stender *et al*., 2000). Like SopE, SopE2 is a GEF, but it interacts efficiently only with CDC42, not RAC1 (Friebel *et al*., 2001), which might explain why it only weakly contributes to host cell invasion *in vitro* (Bakshi et al., 2000; Mirold et al., 2001; Stender et al., 2000) and to gut inflammation *in vivo* (Hapfelmeier et al., 2004). The following genes were also present in all isolates included in the panel: *invA, sitC, spiC, sifA, misL, orfL* and *pipD*. The *invA* gene is located within SPI-1, is highly conserved among all *Salmonella* species (Boyd *et al*., 1997) and is essential for *S*. Typhimurium invasion into cultured epithelial cells (Galán *et al*., 1992). The *sitC* gene is part of the *sit* operon located within SPI-1, which serves as an iron transport system (Zhou *et al*., 1999a).

A second T3SS encoded by a second *Salmonella* Pathogenicity Island (SPI-2) is responsible for translocation of bacterial effector proteins across the membrane of

the *Salmonella*-containing vacuole (SCV), a process that is crucial for intracellular bacterial replication and survival. SpiC protein has been identified as essential for secretion of SPI-2 effector proteins via T3SS-2 and *spiC* mutants are attenuated for virulence during infection of macrophages (Freeman *et al.*, 2002; Uchiya *et al.*, 1999; Yu *et al.*, 2002). SifA is an example of a secreted SPI-2 effector protein and its function is in the elongation of the SCV into tubular structures known as *Salmonella*-induced filaments (Sifs), which are required for maintenance of SCV integrity (Beuzón *et al.*, 2000; Brumell *et al.*, 2002; Stein *et al.*, 1996). Δ*sifA* mutant bacteria lose their vacuolar membrane and are consequently attenuated for virulence in mice (Beuzón *et al.*, 2000; Stein *et al.*, 1996) and unable to replicate within the cytosol of macrophages and fibroblasts (Beuzón *et al.*, 2002).

The gene for *misL* is located within SPI-3 and it encodes an autotransporter protein that, upon transport across the cytoplasmic membrane, serves as an extracellular matrix adhesin involved in bacterial colonisation of the intestine (Dorsey *et al.*, 2005; Tükel *et al.*, 2007). *misL* mutant *S.* Typhimurium have been demonstrated to be attenuated for intestinal colonisation in chicks (Morgan *et al.*, 2004) and pigs (Carnell *et al.*, 2007), suggesting that this gene is a virulence factor. A fourth *Salmonella*-pathogenicity island (SPI-4) that is required for intestinal, but not systemic infection in mice (Kiss *et al.*, 2007; Morgan *et al.*, 2004) was identified by Wong *et al.* (1998), who suggested it carried a type 1 secretion system involved in toxin secretion. The *orfL* gene mapped to the SPI-4 region and was shown to be required for intra-macrophage survival in mice (Wong *et al.*, 1998). Although little else is known about this gene, its presence in all of the isolates in this study confirms the presence of SPI-4. The *pipD* gene, like *sopB*, is encoded on SPI-5 and its protein is structurally similar to dipeptidases from *Lactobacillus* spp., suggesting that it might be a secreted peptidase of *Salmonella* (Wood et al., 1998). SPI-5 is conserved across *Salmonella* serotypes and the presence of *pipD* in all of the isolates suggests that they all possess this pathogenicity island, including S00398, which was negative for *sopB*.

The *iroN* gene is part of the *S. enterica iroA* locus, which along with the *iroBCDE* operon forms a functional unit involved in iron acquisition. Both the *iroBCDE* operon and the *iroN* gene are conserved across all phylogenetic lineages of *S. enterica*, so the fact that *S.* Typhimurium DT193 isolate L00168 was negative by PCR for *iroN* is quite surprising. This gene encodes an outer membrane siderophore receptor

protein and has been speculated to facilitate the growth of *Salmonella* in soil (Bäumler *et al.*, 1998), which would confer a selective advantage in terms of survival in and transmission from the environment. The absence of this gene from the genome of L00168 suggests that this isolate may be attenuated for growth in soil, although other siderophore receptors are present in *S*. Typhimurium whose substrate specificity may overlap with the IroN receptor. The main siderophore, or iron-chelator, produced by *S*. *enterica* and *E. coli* is enterobactin, which enables these bacteria to remove iron from high affinity host binding proteins (Pollack & Neilands, 1970). However, an antimicrobial host protein known as lipocalin-2, which is released by epithelial cells during intestinal inflammation (Raffatellu *et al.*, 2009), binds to enterobactin preventing the use of this siderophore for bacterial iron acquisition during *E. coli* infection (Flo *et al.*, 2004). But *S. enterica* is resistant to lipocalin-2 as shown by work identifying IroN as a mediator of salmochelin uptake (Hantke *et al.*, 2003). Salmochelin is a linear glycosylated derivative of enterobactin that is not bound by lipocalin-2, meaning presence of IroN enables *S. enterica* to acquire iron in the inflamed intestine (Crouch *et al.*, 2008). That isolate L00168 lacks this gene suggests that it may be attenuated for growth in the intestinal lumen of the host during inflammation. However, it is interesting to note that while a *fepA iroN cirA* mutant of *S.* Typhimurium was attenuated in a murine model of infection (Rabsch et al., 2003), there was no significant difference in intestinal colonisation or faecal shedding of the same mutant compared to the wild-type strain in the swine host (Bearson *et al.*, 2008).

The *pefA* gene is located on a 90 kb serotype specific virulence plasmid that is conserved among isolates of *S*. Typhimurium (Friedrich *et al.*, 1993). The *pef* locus encodes plasmid-encoded fimbriae and represents one of 13 fimbrial operons contained in the *S*. Typhimurium genome. Phylogenetic analysis of fimbrial operons in sequenced *Salmonella* genomes revealed that the *pef* locus is only found in the Typhimurium serotype and in a few other serotypes, such as Choleraesuis and Paratyphi C (Yue *et al.*, 2012). Because Pef are not expressed during laboratory culture in LB broth (Humphries *et al.*, 2005; Humphries *et al.*, 2003), little is known about the binding specificity of this adhesin. That said, expression of PefA has been detected in *S.* Typhimurium during *in vivo* infection of mice (Humphries *et al.*, 2005) and during a bovine ligated loop assay (Humphries *et al.*, 2003). Also, the *pef* operon has been shown to mediate bacterial adhesion to murine small intestine

(Bäumler *et al.*, 1996a). However, fimbrial-mediated adhesion to cultured epithelial cells has been mostly attributed to type 1 fimbriae (Bäumler *et al.*, 1996c) and it has been shown that Pef do not play a role in attachment of *S*. Typhimurium to HeLa cells (Misselwitz *et al.*, 2011b). As yet only type 1 fimbriae (Fim) and *Salmonella* atypical fimbriae (Saf) have been identified as playing a role in colonisation of porcine intestines (Althouse *et al.*, 2003; Carnell *et al.*, 2007). So, given that fimbriae have been implicated in regulation of host and tissue-specificity due to their antigenic nature (Yue *et al.*, 2012), it is possible that Pef are not required for infection in the pig. If this were the case, it would go some way to explaining why only one of the six field isolates of *S.* Typhimurium from pigs in this study was positive for the *pefA* gene by PCR. Indeed, Woodward *et al*. (1996) found that out of 133 Typhimurium isolates, only 77 harboured the *pefA* gene, showing that it is not conserved across all isolates of this serotype. Similarly, while none of the 4,[5],12:i:- isolates in this study harboured the *pefA* gene, it was found to be present in the genome of a sequenced 4,[5],12:i:- strain (Yue *et al.*, 2012). Interestingly, the same study revealed that *S*. Typhimurium DT104, another epidemic strain, was also negative for the *pef* locus. Perhaps loss of this fimbrial operon represents a significant adaptation contributing to the success of these epidemic strains. Further work exploring the relationship between fimbrial repertoire and host or niche specialisation is necessary to understand the implications of this gene loss.

The *S.* Typhimurium genome contains 13 fimbrial gene sequences, including type 1 fimbriae (Fim) and plasmid-encoded fimbriae (Pef) and bacterial isolates can express more than one type. It is not possible to determine by TEM alone which fimbriae were present on the DT193 isolates. However, from the PCR virulotyping (Table 2.5) it can be deduced that the fimbriae were not Pef, as both S01299 and L00168 were negative for the *pefA* gene. It is likely that the fimbriae are Fim because FimA has been shown to be the only fimbrial antigen expressed by *S*. Typhimurium after *in vitro* growth in LB broth (Humphries *et al.*, 2003).

Not all of the isolates had visibly detectable fimbriae and not all of the bacterial cells in a culture displaying fimbriae expressed them. This is likely to be due to transcription of some fimbrial operons being regulated by phase variation in *S*. Typhimurium (Nicholson & David, 2000; Norris *et al.*, 1998b; Swenson & Clegg, 1992). A random heritable switching mechanism controls whether fimbrial expression is “on” or “off” and only a proportion of bacteria in a culture will be in the

"on" phase, resulting in heterogenous expression of fimbriae (reviewed by Humphries *et al*. 2001). Therefore, it is possible that the non-fimbriated bacteria observed by TEM may have simply been in the "off" phase of fimbrial expression.

Although the qualitative analysis of proteins secreted into culture media by SDS-PAGE was mostly inconclusive, it is important to mention that most of the predicted secreted proteins were SPI-1 effector proteins belonging to the Sip family. SipA protein binds to actin and inhibits its depolymerisation in order to stabilise it (Galkin *et al.*, 2002; Higashide *et al.*, 2002; McGhie *et al.*, 2001; Zhou *et al.*, 1999b). It has also been shown to contribute to localisation of bacteria in clusters in the vicinity of membrane ruffles, thereby facilitating bacterial uptake by the host cell (Jepson *et al.*, 2001; Perrett & Jepson, 2009; Zhou *et al.*, 1999b). This regulation of ruffle formation is achieved through intricate interplay between SipA, SopE, SopE2 and SopB (Perrett & Jepson, 2009). SipC, another of the predicted secreted proteins, also binds to actin and works in concert with SipA to prevent actin filament disassembly by host factors (McGhie *et al.*, 2001; Myeni & Zhou, 2010). Together with SipB and SipD, SipC forms the SPI-1 translocon required for transfer of the effector proteins across the host cell membrane (Collazo & Galán, 1997b; Kaniga *et al.*, 1995b). Given the importance of the Sip proteins in uptake of *Salmonella* into non-phagocytic host cells, quantification of the secretion of these proteins by the 4,[5],12:i:- and Typhimurium DT193 isolates would be extremely worthwhile for assessment of virulence potential.

In addition to T3SS-1 effector proteins and fimbriae, a key virulence factor that contributes to host cell invasion is the *Salmonella* flagellum. Numerous studies have implicated flagella in successful cellular invasion *in vitro* (Dibb-Fuller *et al.*, 1999; Jones *et al.*, 1992; Jones *et al.*, 1981; La Ragione *et al.*, 2003; Liu *et al.*, 1988) and *in vivo* (Shah *et al.*, 2011; Stecher *et al.*, 2004), although it is generally thought that while flagella-mediated motility accelerates the invasion process, it is not essential (Van Asten *et al.*, 2004). The contribution of motility to virulence is multi-factorial. Firstly, flagellar motility allows *Salmonella* bacteria to approach the host cell surface (Misselwitz *et al.*, 2012), thus increasing the chance of adhesion and invasion. Secondly, motility enables *Salmonella* to outcompete the microbiota in the inflamed intestine through movement towards high-energy nutrients localised at the gut mucosa, which can be utilised for enhanced growth (Stecher *et al.*, 2008). Results of the motility assays revealed that the 4,[5],12:i:- isolates, despite

expressing only one of the two flagellar antigens, were as motile as the control isolate *S*. Typhimurium 4/74, thus suggesting that monophasic expression of flagella does no limit the motility of *Salmonella* DT193 isolates and they should benefit from these motility-based advantages during the early phase of infection. The isogenic Δ*fliC* mutant GC001, created from L00168, was equally as motile as the parent isolate, providing further evidence for this theory. Conversely, *S*. Typhimurium DT193 isolate S01557 had significantly reduced motility compared to the other isolates, suggesting that this isolate may exhibit impaired virulence during intestinal colonisation; this is investigated further in Chapter 3. The Δ*fljB* mutant GC002 was significantly less motile than the parent strain (P = 0.0000) and the other field isolates, except S01557. This was likely to be due to this isolates inability to construct full-length flagella filaments, as shown by TEM (Figure 2.3E). In fact, the Δ*fljB* mutant exhibited an equal sized halo of migration to that of the aflagellate Δ*fliC* Δ*fljB* mutant GC003, suggesting that it was completely immotile.

Previous studies have identified various deletions affecting the *fljAB* operon and flanking genes of Spanish, European and United States 4,[5],12:i:- isolates (Garaizar *et al.*, 2002; Hauser *et al.*, 2010; Hopkins *et al.*, 2010; Laorden *et al.*, 2010; Soyer *et al.*, 2009; Zamperini *et al.*, 2007). The European clonal lineage commonly show a deletion encompassing *fljA*, *fljB* and *hin* (Hauser *et al.*, 2010) as exhibited by the monophasic isolates studied here. Transcription of the *fljA* gene is under direction of the same promoter that controls transcription of *fljB*, and when *fljA* is expressed, transcription of *fliC* is repressed (Aldridge *et al.*, 2006). The absence of both *fljB* and *fljA* in 4,[5],12:i:- isolates provides evidence for selective pressure on these *Salmonella* to express only FliC flagellin. This evidence is further supported by the absence of *hin* in the genome of these isolates. This gene encodes a site-specific recombinase, which controls inversion of the *fljBA* promoter sequence (Haykinson *et al.*, 1996). Without *hin* the *fljBA* promoter, which usually switches orientation to enable flagellar phase variation, is essentially locked into place, thus preventing the bacteria from changing which flagellin it expresses. The combined loss of *hin*, *fljB* and *fljA* presumably results in these isolates being completely unable to express FljB flagellin and unable to switch off production of FliC flagellin. The latter is important because, in the absence of *fljB* it is essential that expression of *fliC* is maintained, otherwise they would not be able to construct functional flagella

filaments and motility would be lost. The transmission electron micrographs (Figure 2.3) confirm that these isolates are indeed able to construct full-length flagella.

Finally, most *Salmonella* serotypes are believed to exhibit a cyclical lifestyle involving a host-associated, infectious phase characterised by motility and an environment-associated, persistent phase characterised by sessility. The latter is typified by aggregation of bacterial cells within an extracellular matrix composed of exopolysaccharide cellulose and curli fimbriae (Austin *et al.*, 1998), otherwise known as a biofilm. The majority of studies investigating the advantages of biofilm formation by *Salmonella* identify resistance to environmental conditions and hence improved transmission between hosts as key fitness traits. For example, *Salmonella* persistence within biofilms on animate and inanimate surfaces in meat-processing plants is widely recognised and poses a significant barrier to control of *Salmonella* contamination and threat to public health (reviewed by Waldner *et al.*, 2012). In addition to the benefits of biofilm formation in the environment, the ability to express this multicellular phenotype within the host has been shown to contribute to successful establishment and maintenance of infection. This has been extensively studied in the case of *S.* Typhi infection, in which formation of biofilms on the surface of human and murine gallstones (Crawford *et al.*, 2010) is associated with a persistent carrier state infection (Gonzalez-Escobedo *et al.*, 2011). Similarly, *S.* Typhimurium has also been shown to form biofilms on gallstones, murine gallbladder epithelia, human epithelial cells and chicken intestinal epithelia (Gonzalez-Escobedo & Gunn, 2013; Ledeboer *et al.*, 2006; Prouty & Gunn, 2003). However, biofilm production of *S.* Typhimurium within the host and its role in pathogenicity is not well characterised. Indeed, extracellular colonisation of porcine tonsils *in vivo* has been shown to be independent of biofilm formation (Van Parys *et al.*, 2010), suggesting that expression of this phenotype is relevant to only certain host tissues.

Regardless, results pertaining to the biofilm-forming abilities of the *Salmonella* isolates in this study may give some clues as to their virulence potential. The results suggest that all of the isolates are able to adhere to polystyrene and glass during incubation at room temperature and 37°C. However, they exhibit differences in their rates of biofilm formation and in the temperatures favoured by each isolate for expression of this phenotype. Monophasic isolate S01299 produces a biofilm faster than the other isolates when grown at 37°C. Conversely, monophasic isolate

S03554 and biphasic isolates S01557 and 4/74, display a slower rate of biofilm production, with a preference for room temperature. *S*. Enteritidis isolate P125109 has a fast initial rate of biofilm formation, with no preference for temperature, but then its accumulation of biofilm does not increase with added incubation time.

That 4,12:i:- isolate S01299 and *S.* Enteritidis P125109 both form biofilms on polystyrene and glass rapidly (within 24 h) at 37°C suggesting that they could potentially demonstrate this phenotype on internal host tissues. Formation of bacterial aggregates on host tissues could aid colonisation and invasion during early infection and/or contribute to chronic infection. Conversely, 4,5,12:i:- isolate S03554 and phase variable *S*. Typhimurium isolates S01557 and 4/74 show evidence of biofilm formation on polystyrene and glass at ~25°C over extended periods of time, suggesting that these isolates would be better adapted to survival outside of the host and could consequently have a selective advantage due to increased likelihood of transmission to other hosts. Perhaps these isolates might also demonstrate enhanced persistence on surfaces relevant to the meat-processing environment, such as stainless steel; it would be interesting to investigate this further. Finally, that all three of the monophasic DT193 isolates showed the ability to produce biofilms on glass and polystyrene is consistent with a recent study by Seixas and colleagues (2014) who found that the majority of 133 *Salmonella* 4,[5],12:i:- isolates collected in Portugal from clinical, environmental and animal origins, were able to form biofilms on both plastic and glass surfaces. Interestingly, the same study revealed that the biofilm production ability of Portuguese 4,[5],12:i:- isolates increased between the years of 2006 and 2011, suggesting an improved aptitude to persist in the environment, which might partly explain the ecological success of this strain.

Together, the data presented in this chapter show that isolates of *Salmonella* 4,[5],12:i:- and Typhimurium belonging to phage type DT193 and originating from pigs possess a heterogeneous repertoire of virulence-related phenotypes. This suggests that the reason behind their epidemic spread is not unifactorial. In the assays conducted in this chapter, no clear-cut differences between monophasic and flagellar phase variable isolates were identified. Therefore, failure to express the *fljB* gene does not appear to impact the fundamental *in vitro* phenotypic traits of *S*. Typhimurium, at least in the small panel of isolates studied here.

3 Host-Pathogen Interaction During Early Infection

3.1 Introduction

At least 95 genes are required by *Salmonella* Typhimurium for successful colonisation of the intestine in the pig host (Carnell *et al.*, 2007). Comparison of these genes with those identified in similar *in vivo* studies, using signature-tagged transposon *S.* Typhimurium mutants, in mice, chickens and calves (Morgan *et al.*, 2004; Tsolis *et al.*, 1999b) revealed that this serotype uses both conserved and host-specific colonisation factors. These findings emphasise, firstly, that colonisation of the intestine is an important enough step in *Salmonella* infection for it to invest a significant proportion of its genome to and secondly, that it is important to study colonisation on a host-by-host basis given that different genes are required in different hosts.

The intestinal epithelium represents a physical barrier against enteropathogenic bacteria and the adhesion to and invasion of this surface by *Salmonella* is considered to be a key indicator of virulence (Boyen *et al.*, 2006c; Hurley & McCormick, 2003). Specialised membranous epithelial (M) cells with the capacity to transcytose and present antigens are known to be targets of *Salmonella* invasion in the distal intestine (reviewed by Jepson & Clark, 2001). *Salmonella* is capable of actively invading non-phagocytic cells, such as M cells and regular intestinal epithelial cells, through manipulation of the actin cytoskeleton of the host cell. Prior to invasion, *S.* Typhimurium adheres to the mucosal surface, a process that can be either reversible or irreversible, the latter of which involves the SPI-1 T3SS (Lara-Tejero & Galán, 2009; Misselwitz *et al.*, 2011b). Irreversible binding of the bacterium to a host cell commits it to invasion, following translocation of SPI-1 effectors and consequent host-cell membrane ruffling and engulfment of the bacterium by macropinocytosis. Once *Salmonella* crosses the intestinal epithelium it encounters an array of phagocytic immune cells, including dendritic cells, macrophages and neutrophils (Rydström & Wick, 2007). It is within these cells, and within IECs, that *Salmonella* establishes its intracellular niche. SPI-2-mediated formation of the SCV and modulation of membrane trafficking enhances the intracellular survival and replication of *Salmonella* (Chakravortty *et al.*, 2002; Holden, 2002; Vázquez-Torres *et al.*, 2000b; Vázquez-Torres & Fang, 2001b;

Vázquez-Torres *et al*., 2001). Because of its association with systemic spread from the subepithelium, survival and replication within macrophages is also considered to be a marker of *Salmonella* virulence.

Most research concerning *Salmonella*-host interactions is derived from *in vivo* and *in vitro* murine models. For example, the streptomycin-treated mouse model of *Salmonella* diarrhoea has played a crucial role in studying the molecular pathogenesis of *Salmonella* and the host mucosal immune response (reviewed by Kaiser *et al*., 2012). Ligated ileal loop models in calves, pigs and rabbits have also been instrumental to the study of *Salmonella* colonisation and early innate immune responses of the host (Bolton *et al*., 1999; Boyen *et al*., 2006b; Boyen *et al*., 2009; Wallis *et al*., 1989). *In vitro* research into this bacterium's interactions with intestinal epithelial cells has involved a wide range of cell lines, including Madin-Derby canine kidney (MDCK) cells and human T84, Caco-2, HT29 and HeLa cells. The use of cell lines of porcine origin is comparatively rare, mainly due to a lack of availability of suitable lines. Only three porcine intestinal epithelial cells lines have been characterised: IPI-2I, IPEC-1 and IPEC-J2 (Gonzalez-Vallina *et al*., 1996; Kaeffer *et al*., 1993; Koh *et al*., 2008; Schierack *et al*., 2006). Instead, the majority of *Salmonella* pathogenicity studies in swine have been performed *in vivo*. While the value of animal infection studies is unquestionable, it must be borne in mind that their use is often costly and time-consuming and requires high numbers of experimental animals to achieve meaningful results. Additionally, given the complexity of the coordinated interplay between different mediators in the gut during *Salmonella* infection, *in vivo* experiments tend to be more difficult to interpret than ones using cultured cells. Therefore, porcine *in vitro* models have an important place in initial studies of the infection of *Salmonella* strains of interest in the context of the pig, before confirmation of findings in the live animal (Boyen *et al*., 2009). Of course, caution should always be exercised when extrapolating data obtained *in vitro* to assumptions about infection in the live host, or in other species.

As pigs are the major reservoir of *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates (Hopkins *et al*., 2010) and current control methods of *Salmonella* in pigs rely largely on hygienic measures with no guarantee of success, the characterisation of infection with these strains in a pig-based model was deemed to be of value. Identification of the virulence of these strains in pigs could inform future vaccine design and/or additional pre-harvest control measures for the limitation of their

perpetuation in this host species. Also, it was hoped that comparison of the *in vitro* behaviour of monophasic and biphasic isolates with intestinal epithelial cells of this important host might reveal explanations for the recent emergence and success of strains expressing only one flagellar antigen, instead of two. Therefore, the adhesion and invasion levels of pig-associated field isolates of *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates in an IPEC-1 cell culture model were investigated. Invasiveness into Caco-2 cells was also determined, as an indicator of potential virulence in humans. Finally, their replicative power in murine RAW264.7 macrophages was determined as a basic indicator of these isolates' potential to cause systemic infection. Results are discussed in relation to expression of the SPI-1 virulence genes.

3.2 Materials and Methods

3.2.1 *Salmonella* isolates

The isolates of bacteria used throughout this study are detailed in Chapter 2 (Tables 2.1 and 2.4) and Table 3.1. The isolates of pig origin were kindly donated by the Animal Health Veterinary Laboratories Agency (Surrey, UK) who isolated them from pig faeces on farms in Great Britain.

3.2.2 Bacterial culture conditions

All *Salmonella* isolates were stored on Microbank™ cryoprotective beads (Pro-Lab Diagnostics Inc., UK) at -80°C. Using aseptic technique, isolates were streaked from frozen stocks onto nutrient agar and incubated aerobically for 24 h at 37°C. Stationary phase cultures were prepared by inoculating 10 ml standard Miller formulation Luria-Bertani (LB) broth with 2-3 colonies from a nutrient agar plate using a sterile loop. Cultures were incubated overnight for 16-18 h at 37°C, 150 rpm in an orbital shaker. Late-logarithmic phase cultures were achieved by diluting stationary phase cultures 1:100 (v/v) into fresh LB broth followed by incubation at 37°C, 150 rpm for a further 3.5 hours.

3.2.3 Cell culture

3.2.3.1 IPEC-1

The IPEC-1 cell line is an undifferentiated intestinal epithelial line derived from the small intestine of a neonatal unsuckled piglet (Gonzalez-Vallina *et al.*, 1996). This line was a kind gift from Dr. Tristan Cogan, University of Bristol. Cells were maintained in 75 cm^2 plastic cell culture flasks (Cellstar, Greiner Bio-One Ltd., UK) at 37°C in a humidified atmosphere of 5% CO_2. They were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Life Technologies Ltd., UK) supplemented with 5% foetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium (ITS Premix; BD Biosciences, UK) and 0.5 ng/ml human epidermal growth factor, hereafter referred to as IPEC-1 medium. Continuous cultures of IPEC-1 cells were maintained by passaging at a 1:3 or 1:5 ratio. Cell passage numbers were kept within the range of 128 to 140. In preparation for subculture, the culture medium was removed and the cell layer was rinsed twice with PBS. 5 ml trypsin-EDTA was added and the flask was incubated at 37°C for 5 to 15 minutes to release the cells from the surface of the flask. The required volume of IPEC-1 medium was added to inactivate the trypsin and the cells were aspirated by gentle pipetting. Appropriate aliquots of the cell suspension were added to new culture vessels and the volume was brought up to 20 ml with fresh, pre-warmed IPEC-1 medium.

3.2.3.2 Caco-2

The Caco-2 cell line (ATCC® HTB-37) is a human colonic epithelial line (Fogh *et al.*, 1977) derived from a colon carcinoma. This cell line forms polarised monolayers in culture and differentiates into cells with intestinal epithelial enterocyte morphology (Pinto *et al.*, 1983). Caco-2 cells were maintained under the same conditions as above, in standard DMEM (Life Technologies Ltd., UK) supplemented with 10% foetal calf serum, 1% L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acid solution, hereafter referred to as complete DMEM. Continuous cultures of Caco-2 cells were maintained by passaging at a ratio of 1:3 to 1:6, with renewal of medium 1 to 2 times per week. Subculturing was performed as described for IPEC-1 cells (Section 3.2.3.1). Cell passage numbers were kept within the range of 45 to 55.

3.2.3.3 RAW264.7

RAW264.7 (ATCC® TIB-71) is a murine monocytic macrophage cell line (Raschke *et al.*, 1978). These cells were cultured and maintained in complete DMEM in 75 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. Subcultures were prepared by removal of all but 10 ml medium from the flask and release of the cells from the plastic with a cell scraper (Corning, Appleton Woods Ltd., UK). The cell suspension was then aspirated by gentle pipetting and appropriate aliquots were added to new culture vessels. RAW264.7 cells were passaged every 2 to 3 days at a ratio of 1:3 to 1:6. Cell passage numbers were kept within the range of 80 to 95.

3.2.4 Infection of cultured IPEC-1 cells

When confluent, IPEC-1 cells were prepared for *Salmonella* infection to determine bacterial adhesion and invasion. Spent medium was removed from the culture flask and cells were washed twice with warm, sterile PBS and incubated with 5 ml trypsin-EDTA. IPEC-1 medium (5 ml) was then added and 10-20 µl of cell suspension was transferred to a haemocytometer after gentle aspiration with a pipette to disrupt cell clumps. The total number of cells per millilitre of medium was calculated following enumeration of the number of cells in each of the 4 x 4 corner squares of the haemocytometer. The mean number of cells per corner square was multiplied by 1 x 10^4 to compensate for the depth of the haemocytometer.

For assessment of adhesion and invasion by differential antibody staining, IPEC-1 cells were seeded onto sterile 13 mm glass coverslips (Thickness #1; VWR International Ltd., UK) in 12-well tissue culture plates at a density of 1 x 10^5 cells per well and grown at 37°C in a humidified atmosphere (5% CO_2) for 2 days. Prior to infection, cells were washed three times with warm, sterile PBS and overlaid with 1 ml pre-warmed antibiotic-free IPEC-1 media. After equilibration in this medium at 37°C, 5% CO_2, 50 µl late-log phase bacterial cultures were added to achieve an approximate multiplicity of infection (MOI) of 10 bacteria per IPEC-1 cell. Cells were then incubated for 15 or 30 min at 37°C in air. After the appropriate incubation time, coverslips were removed from the wells of the tissue culture plate, washed with moderate agitation in PBS and fixed overnight in 2% paraformaldehyde (PFA) at 4°C.

3.2.5 Differential immunofluorescent staining for detection of adhered and invaded *Salmonella*

Following infection and fixation, *Salmonella* were immunolocated by differential antibody staining according to the method described by Perrett & Jepson (2007). Coverslips were washed in room temperature PBS and then incubated with 50 µl goat anti-*Salmonella* CSA-1 antibody (Insight Biotechnology Ltd., UK) diluted 1:200 (v/v) in PBS for 45 min at room temperature. Adhered extracellular bacteria were labelled with 50 µl rabbit anti-goat Alexa Fluor 488 (Life Technologies Ltd., UK) diluted 1:200 (v/v) in PBS for 45 min at room temperature. IPEC-1 cells were then permeabilised for 20 min with 0.3% (v/v) Triton-X100 (Sigma-Aldrich Ltd., UK) in PBS and washed with PBS. Goat anti-*Salmonella* CSA-1 antibody was applied again at the same concentration as before and rabbit anti-goat Alexa Fluor 594 (Life Technologies Ltd., UK) diluted 1:200 (v/v) in PBS was added to the cells for 45 min at room temperature to label all intracellular and extracellular bacteria. Coverslips were washed extensively with PBS between all staining steps. Finally, coverslips were mounted onto microscope slides, cell-side facing down, with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Ltd., UK) to label IPEC-1 nuclei.

Counts of bacteria (adhered and total) and IPEC-1 cells were performed using a Nikon Eclipse E80i fluorescence microscope with oil-immersion lens (100x) during which 10 randomly selected fields were analysed per coverslip, amounting to at least 200 IPEC-1 cells per sample. The total number of cell-associated (adhered and invaded) *Salmonella* per IPEC-1 cell for each sample was calculated by dividing the total number of bacteria by the number of IPEC-1 cells. To calculate the number of invaded *Salmonella* per IPEC-1 cell, the number of adhered bacteria was subtracted from total cell-associated bacteria and divided by the number of IPEC-1 cells.

3.2.6 Confocal microscopy

Immunofluorescent images were acquired in the University of Liverpool's Centre for Cell Imaging, using a Zeiss LSM 510 META Two-Photon Laser Scanning Confocal Microscope. Alexa Fluor 488 was detected using an argon (488 nm) laser, 565 nm dichroic and 500-530 nm bandpass filter. Alexa Fluor 594 was detected using a

DPSS (561 nm) laser, 565 nm dichroic and 575 nm long pass filter. DAPI was detected using a SpectraPhysics MaiTai Ti:sapphire laser tuned to 700 nm, a 490 nm dichroic, and 435-485 nm bandpass filter. Images were analysed using Imaris software (Bitplane). Image acquisition and analysis was performed by Dr. Janine Coombes, University of Liverpool.

3.2.7 Autoaggregation assay

Bacterial autoaggregation is a process whereby individual bacteria accumulate together which, when cultured in static liquid medium, can cause them to settle to the bottom of the suspension (Fletcher, 1987). To determine whether the *Salmonella* isolates displayed autoaggregation, the settling kinetics of the bacteria were monitored over time according to the method described by Wells *et al.* (2008). Overnight cultures of *Salmonella* were subcultured 1:100 (v/v) into 10 ml fresh, pre-warmed LB broth and grown for a further 4 hours at 37°C with shaking at 150 rpm. The cultures were vortexed for 10 s and left to stand at room temperature or 4°C for 3 hours. At 30 min intervals, a 200 µl sample was taken from approximately 0.5 cm below the liquid-air surface and transferred to a microtitre plate (flat-bottomed; Greiner Bio-One Ltd., UK) for determination of OD_{600} with a Multiskan FC microplate reader (Thermo Scientific, UK). A drop in optical density over time would be indicative of autoaggregation and consequent settling of the bacteria.

3.2.8 Determination of SPI-1 gene expression

3.2.8.1 Construction of S. Typhimurium PprgH-gfp$^+$ reporter strains

In order to investigate expression of the SPI-1 gene *prgH* in the *Salmonella* isolates, single-copy chromosomally integrated P*prgH-gfp*$^+$ reporter strains were created. The plasmids and *Salmonella* strains used for this work are detailed in Table 3.1. The P*prgH-gfp*$^+$ reporter construct (pSH04) was transduced by bacteriophage P22 (see Chapter 2, Sections 2.2.5.1-2.2.5.3) from SH001 into 4,[5],12:i:- isolates S01299 and S03554 and Typhimurium isolates L00168 and S01557.

Table 3.1 Plasmids and *Salmonella* strains used in P*prgH-gfp*$^+$ expression work.

	Genotype	Antibiotic resistance	Reference
Plasmid			
pSH04	P*prgH-gfp*$^+$	KAN	Humphrey *et al.*, 2011
Strains			
SH001	SL1344; P*prgH-gfp*$^+$	KAN	Humphrey *et al.*, 2011
JH3016	SL1344; P*rpsM-gfp*$^+$	CM	Hautefort *et al.*, 2003
GC004	S01299 4,12:i:- DT193 P*prgH-gfp*$^+$	T, A, S, SU, KAN	This study
GC005	S03554 4,5,12:i:- DT193 P*prgH-gfp*$^+$	T, KAN	This study
GC006	S01557 DT193 P*prgH-gfp*$^+$	T, KAN	This study
GC007	L00168 DT193 P*prgH-gfp*$^+$	T, A, S, SU, KAN	This study

Abbreviations: KAN, kanamycin; T, tetracycline; A, ampicillin; S, streptomycin; SU, sulfamethoxazole; CM, chloramphenicol.

3.2.8.2 Quantification of GFP-expressing Salmonella *by flow cytometry*

Overnight cultures of *Salmonella* were subcultured 1:100 (v/) into fresh LB broth and grown for 6 h at 37°C with shaking at 150 rpm. At hourly time intervals, a 0.5 ml sample of bacterial culture was collected and centrifuged at 3300 x *g* for 2 min. The supernatant was discarded and the pellet was resuspended in 0.5 ml 2% (w/v) PFA in PBS. Following fixation overnight at 4°C, samples were diluted 1:50 (v/v) in PBS that had been filtered through a 0.22 μm syringe filter unit (Millopore Ltd., UK). The samples were analysed using a BD Accuri C6 flow cytometer (BD Biosciences, UK) for determination of the proportion of the population expressing green fluorescent protein (GFP). Fluorescence gates were set using *gfp* negative *S*. Typhimurium 4/74 and JH3016, in which *gfp* is constitutively expressed under control of the *rpsM*

promoter. All samples were analysed using the same fluorescence gate settings and *gfp*$^{+}$ *Salmonella* were expressed as a percentage of the total population. 100,000 events were recorded per sample, using an FSC-H primary threshold of 3,500 to eliminate noise.

3.2.9 Minimum inhibitory concentration (MIC)

To ensure that the colistin-protection assays for invasion were conducted correctly, it was first necessary to determine the minimum concentration of the antibiotic that inhibited visible growth of the *Salmonella* isolates on agar.

3.2.9.1 Correlation between optical density and bacterial cell number

Cultures of *Salmonella* were grown overnight in 10 ml LB broth in an orbital incubator set to 37°C, 150 rpm. The optical density at 600 nm (OD_{600}) of each culture was determined using a Multiskan FC Microplate reader (Thermo Scientific, UK). Samples from each culture were then serially diluted in sterile PBS and plated onto nutrient agar. Agar plates were incubated at 37°C for 20-24 h, after which the number of colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 50 colonies. It was calculated that overnight cultures should be diluted 1:10 (v/v) in fresh LB broth to obtain cultures containing 1 x 10^8 CFU/ml, as required for plating onto agar for MIC determination (Wiegand *et al.*, 2008).

3.2.9.2 Agar dilution

Diagnostic sensitivity test (DST) agar (Oxoid Ltd., UK) was prepared according the manufacturer's instructions and cooled to 50°C. A 10 mg/ml stock solution of colistin sulphate salt (Sigma-Aldrich Ltd., UK) was prepared in dH_2O. This stock solution was diluted 1:10 (v/v) in dH_2O to achieve a 1 mg/ml solution, which was in turn diluted 1:10 (v/v) to achieve a 0.1 mg/ml solution. Appropriate volumes of the three colistin stock solutions were dispensed into sterile 30 ml Universal containers according to the antibiotic dilution chart (Table 3.2). 25 ml DST agar was added to each Universal container of colistin, swirled to mix and poured into petri dishes. A control plate with no colistin was also prepared. Once the agar had set the plates were stored overnight at 4°C.

Overnight cultures of the *Salmonella* isolates adjusted to 1 x 10^8 CFU/ml were diluted again 1:10 (v/v) into wells of a 96-well microtitre plate. A multichannel pipette set to 1 μl was used to deliver spots of each bacterial culture onto the agar plates, starting with the control plate and working upwards from the lowest to the highest concentration plate. Inoculum spots were left to dry at room temperature before the plates were incubated at 37°C for 16-20 h. Plates were observed and the agar plate containing the lowest concentration of colistin that inhibited visible growth of each isolate was determined.

Table 3.2 Colistin dilution chart for agar dilution method of MIC determination.

Colistin stock concentration (mg/ml)	Volume of colistin stock solution (μl)	Final concentration when adding 25 ml agar
10	640	256
10	320	128
10	160	64
10	80	32
10	40	16
1	200	8
1	100	4
1	50	2
0.1	250	1
0.1	125	0.5
0.1	62.5	0.25

Adapted from Wiegand *et al*. (2008).

3.2.10 Colistin-protection assay

For determination of *Salmonella* invasion into Caco-2 and RAW 264.7 cells, colistin-protection assays were performed. Caco-2 cells were seeded at a density of 2 x 10^5 cells per well in antibiotic-free complete DMEM in a 24-well tissue culture plate and grown at 37°C in a humidified atmosphere (5% CO_2) for 2 days to achieve a final density of 1 x 10^6 cells per well. Overnight cultures of *Salmonella* were subcultured 1:100 (v/v) into fresh pre-warmed LB broth and incubated for a further 3.5 h at 37°C, 150 rpm to achieve late log-phase cultures. Caco-2 cells were infected with bacterial cultures at an MOI of 10 bacteria per Caco-2 cell. Serial dilutions from the

inoculum were plated onto nutrient agar for calculation of the actual number of bacteria inoculated. Bacteria were brought into contact with the host cells by mild centrifugation at 165 x *g* for 5 min, to synchronise infection. The infected cells were incubated for 1 h at 37°C, 5% CO_2, after which the medium was aspirated from the wells and the cells were overlaid with 1 ml complete DMEM containing 150 μg/ml colistin sulphate. Colistin was chosen because two of the bacterial strains were resistant to the more commonly used gentamicin and this antibiotic has previously been shown to be effective at killing extracellular bacteria (Bergeron *et al.*, 2009; Kusters *et al.*, 1993). The cells were incubated again at 37°C, 5% CO_2 for 90 min to kill extracellular bacteria. Following aspiration of the media, the cells were washed 4 times with PBS to remove any residual antibiotic and the cells were lysed with 1 ml PBS containing 1% (v/v) Triton-X100 for 5 min at room temperature. Each well was vigorously mixed with a pastette before serial dilution of each sample across a microplate and plating onto nutrient agar for enumeration of CFU after 24 h incubation at 37°C.

RAW264.7 cells were seeded at a density of 2 x 10^5 cells per well in antibiotic-free complete DMEM in 24-well tissue culture plates and grown at 37°C, 5% CO_2 for 24 h to achieve a final density of 5 x 10^5 cells per well. RAW264.7 cells were infected with stationary phase cultures of *Salmonella* at an MOI of 10 and plates were centrifuged at 165 x *g* for 5 min. Infection was allowed to proceed for 30 min at 37°C, 5% CO_2 before the media was removed and cells were overlaid with complete DMEM containing colistin. For determination of invasion/phagocytation of *Salmonella* into the macrophages, colistin at a concentration of 150 μg/ml was added to tissue culture plates set up in parallel for 90 min at 37°C, 5% CO_2 before proceeding with the assay as described for Caco-2 cells. For determination of replication/persistence within the macrophages, colistin was added at a concentration of 20 μg/ml for 24 h to inhibit growth and reinvasion of any bacteria released by lysed IPEC-1 cells (Jones *et al.*, 2001), before continuing with the assay from the addition of complete DMEM containing 150 μg/ml for 1 h.

3.2.11 Statistical analysis

Statistical analyses were performed using MiniTab software, version 16. Mean values and standard deviations were calculated and isolates were compared using one-way ANOVA, unless stated otherwise. Differences were considered significant when $P<0.05$. In the event that a significant difference was found, the Tukey method of post-hoc multiple comparisons was performed.

3.3 Results

3.3.1 Adhesion to and invasion of IPEC-1 cells

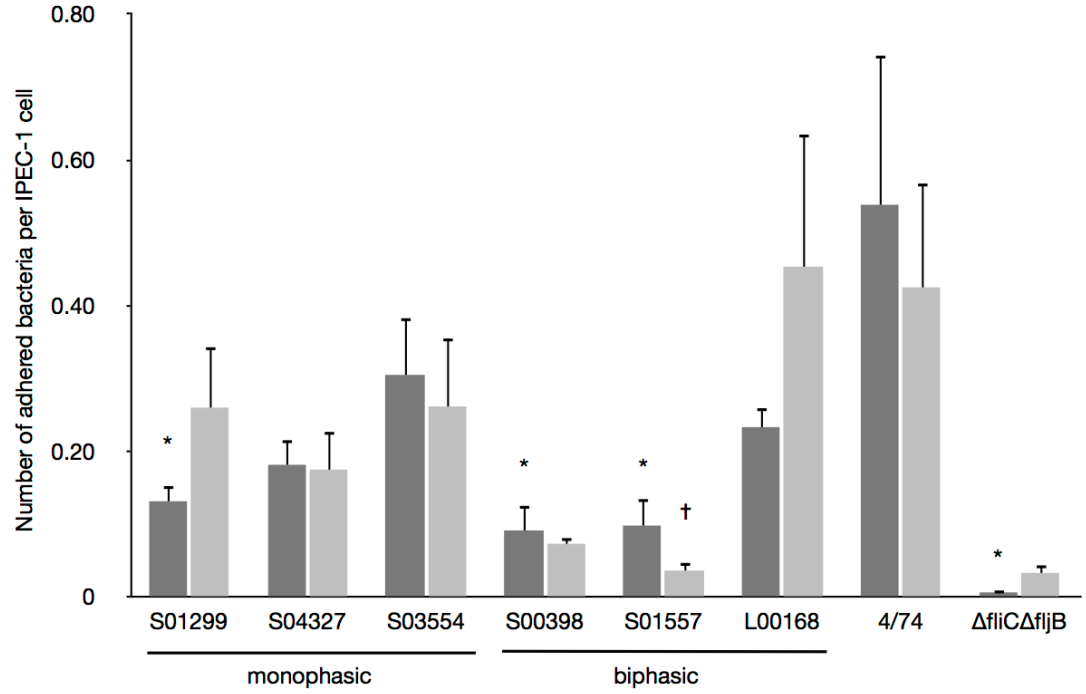
Invasion of *Salmonella* into intestinal epithelial cells following SPI-1 T3SS-mediated irreversible binding and stimulation of membrane ruffles is a crucial event during colonisation of the gut mucosa. Analysis at the single cell level of the adhesiveness and invasiveness of *Salmonella* isolates in an IPEC-1 model of infection was performed (Figure 3.1). After 15 min infection, all isolates adhered to and invaded IPEC-1 cells at a ratio of 0.6 bacteria per host cell or less. By 30 min, there was little to no change in the number of adherent bacteria for most isolates, but the number of intracellular bacteria increased 1.6 to 3.3-fold for all isolates except S01557, which had fewer intracellular bacteria at 30 min than at 15 min. Researchers using the same method of determining invasion per cell of *Salmonella* strains into MDCK cells reported a slightly higher level of invasion after 15 min for *S.* Typhimurium strains SL1344, S1579/94 and F98 (~1.5 bacteria per cell), but a similar level of invasion for *S*. Typhimurium 12023 of 0.5 bacteria per cell (Clark *et al*., 2011). *S.* Typhimurium strain SL1344 used in their study should be approximately comparable to the control strain used in this study, 4/74; the observed differences in invasion could be a consequence of an MOI of 50 being used by Clark *et al*. compared to one of 10 being used here. Therefore, the adhesion and invasion levels observed in this study are approximately consistent with previous work by others.

The effect of monophasic expression of flagella on the ability of *Salmonella* to invade IPEC-1 cells was of interest, given that flagella-derived motility is crucial for bringing *Salmonella* into contact with the intestinal epithelium (Misselwitz *et al*., 2012). The aflagellate mutant GC003 (Δ*fliC* Δ*fljB*) was attenuated in adhesion and

invasion of IPEC-1 cells, compared to the parent strain L00168 (Figure 3.1). This corroborates what others have found, that flagella-based motility is not essential for invasion but accelerates the process (Dibb-Fuller *et al.*, 1999; Jones *et al.*, 1992; La Ragione *et al.*, 2003; Liu *et al.*, 1988; Van Asten *et al.*, 2004). Fluorescence microscopy revealed that *S.* Typhimurium DT193 isolates S00398 and S01557 (both biphasic) were significantly less adhesive (Figure 3.1A) and invasive (Figure 3.1B) than the positive control strain *S.* Typhimurium 4/74 ($P < 0.05$). However, a third biphasic isolate, L00168, showed comparable adhesive and invasive ability to 4/74. The adhesive and invasive ability of the monophasic strains of DT193 were not significantly different to that of 4/74 and no significant differences were detected between the monophasic and biphasic isolates. These results were seen after infection times of both 15 and 30 min.

During microscopic analysis it was noted that the distribution of bacteria was not homogenous through the cell monolayers. Rather, interactions between epithelial cells and bacteria were confined to just 13% of IPEC-1 cells, calculated from the total number of IPEC-1 cells counted per coverslip and the number of those cells with bacteria (Figure 3.2). This proportion was not statistically different across monolayers infected with the different isolates. Other studies have also shown that invasion occurs in only a small fraction of mucosal cells during infection in the mouse (Hapfelmeier *et al.*, 2005) and bovine ligated loop (Reis *et al.*, 2003). It was also noted that while many IPEC-1 cells had just 1 or 2 bacteria attached or within them, there were also cells that appeared to be experiencing “hyperinvasion” of bacteria (Figure 3.3). A maximum number of 10 bacteria per host cell was set for ease of counting when analysing the distribution of bacteria (but not when counting for adhesion and invasion). However, in reality the number of bacteria involved in these hyperinvasion events often reached up to and above 100. Autoaggregation assays performed with each isolate showed that none exhibited autoaggregation (Figure 3.4), suggesting that hyperinvasion was not due to clumping of bacterial cells during culture. The hyperinvasion phenomenon was most often seen during infection with S01299 (4,12:i:-), S03554 (4,5,12:i:-) and 4/74. It appears that the isolates that exhibited the greatest invasive ability were the same ones that demonstrated “hyperinvasion” (Figure 3.1B and Figure 3.3B). However, even isolates that were relatively less invasive showed evidence of hyperinvasion (S01557, Figure 3.3C).

A.


Number of adhered bacteria per IPEC-1 cell
0.80
0.60
0.40
0.20
0
S01299
S04327
S03554
S00398
S01557
L00168
4/74
ΔfliCΔfljB
monophasic
biphasic

B.

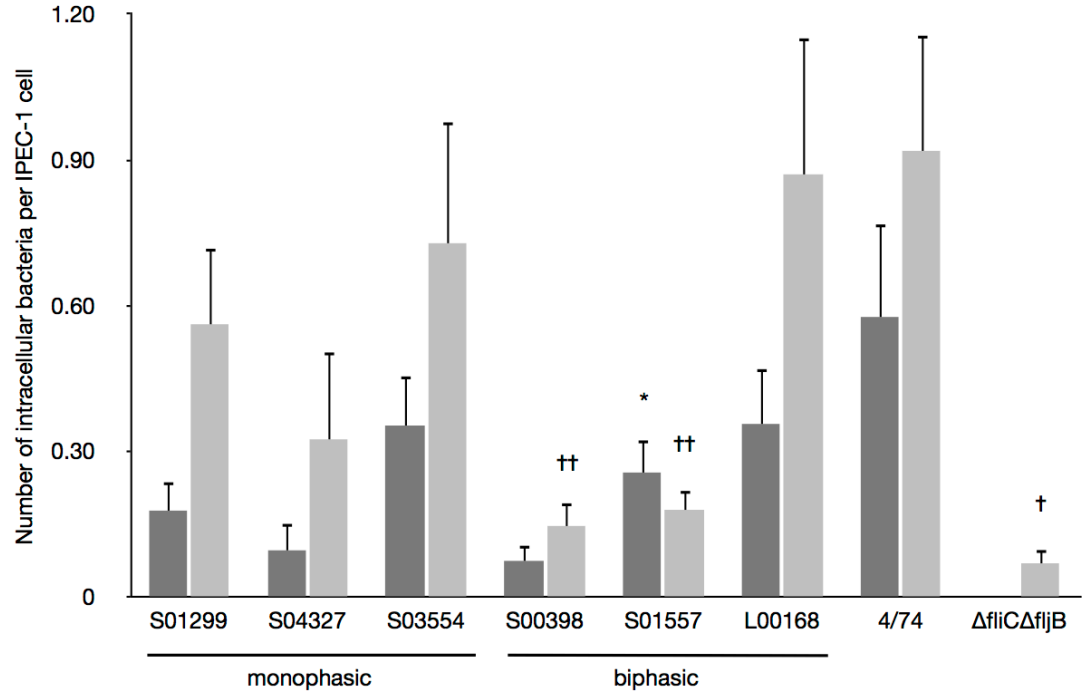

Number of intracellular bacteria per IPEC-1 cell
1.20
0.90
0.60
0.30
0
S01299
S04327
S03554
S00398
S01557
L00168
4/74
ΔfliCΔfljB
monophasic
biphasic

Figure 3.1 Comparison of adhesion and invasion of *Salmonella* isolates during IPEC-1 cell model of early infection.

IPEC-1 cells grown on glass coverslips were infected with *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and *S.* Typhimurium 4/74 at an MOI of 10 for 15 (■) or 30 (■) min. Adhesion (**A**) and invasion (**B**) was assessed by differential antibody staining in three independent experiments with each experiment performed in duplicate. Results are mean ± SEM, where at least 200 IPEC-1 cells were assessed per coverslip from 10 randomly selected fields of view. Statistically significant differences from the positive control strain 4/74 at 15 min (*) and 30 min (†) are indicated as follows: *, $P < 0.05$; †, $P < 0.05$, ††, $P < 0.01$.

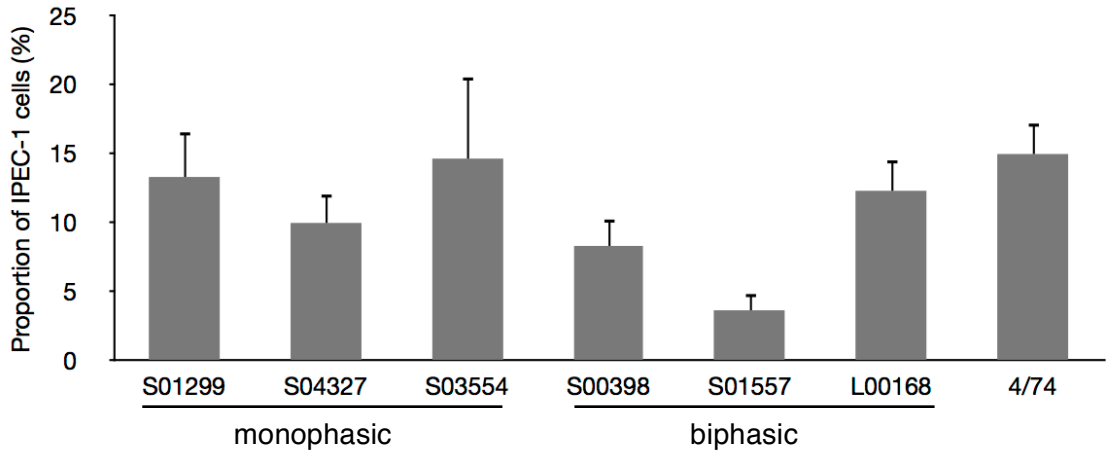
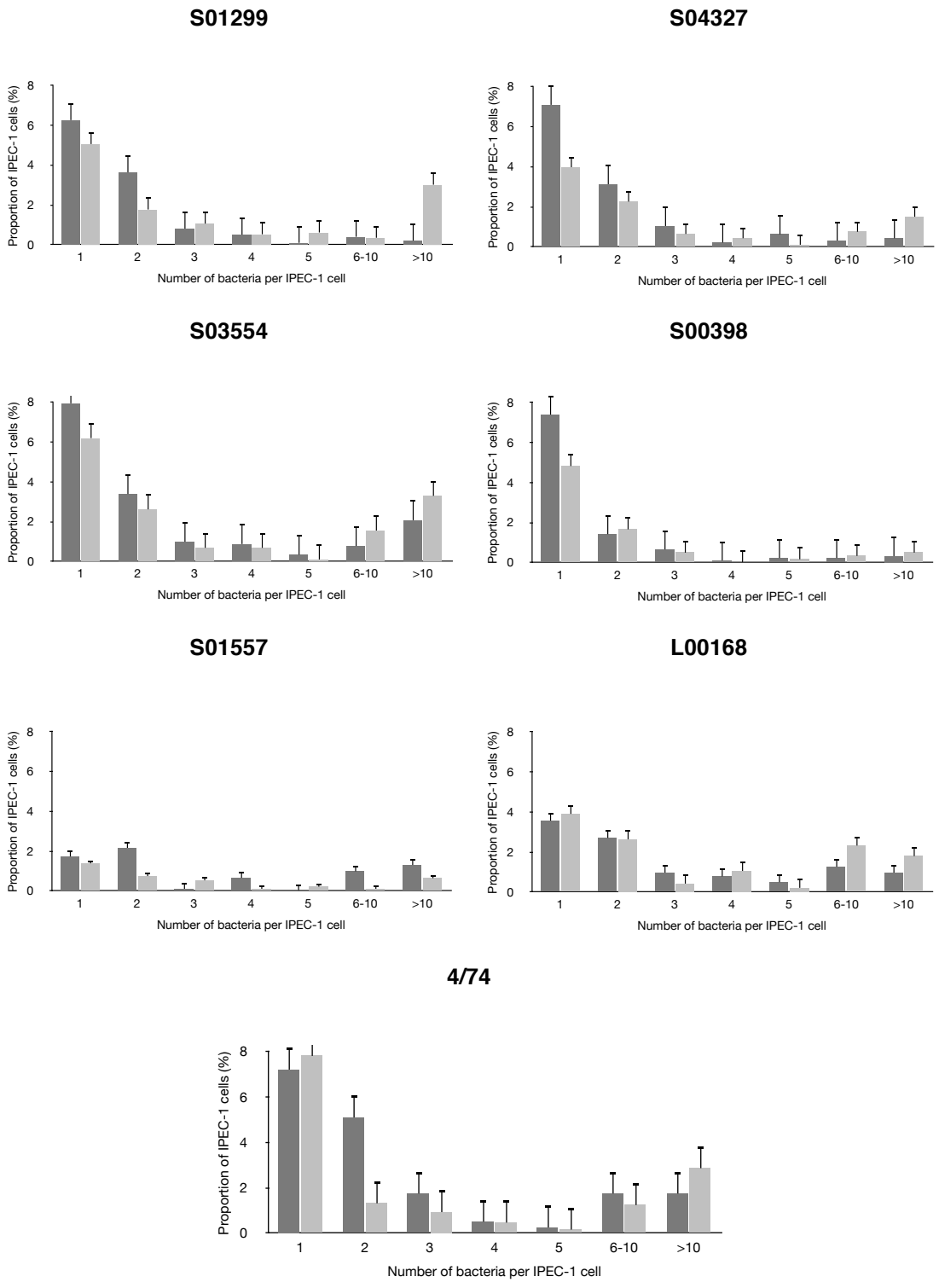


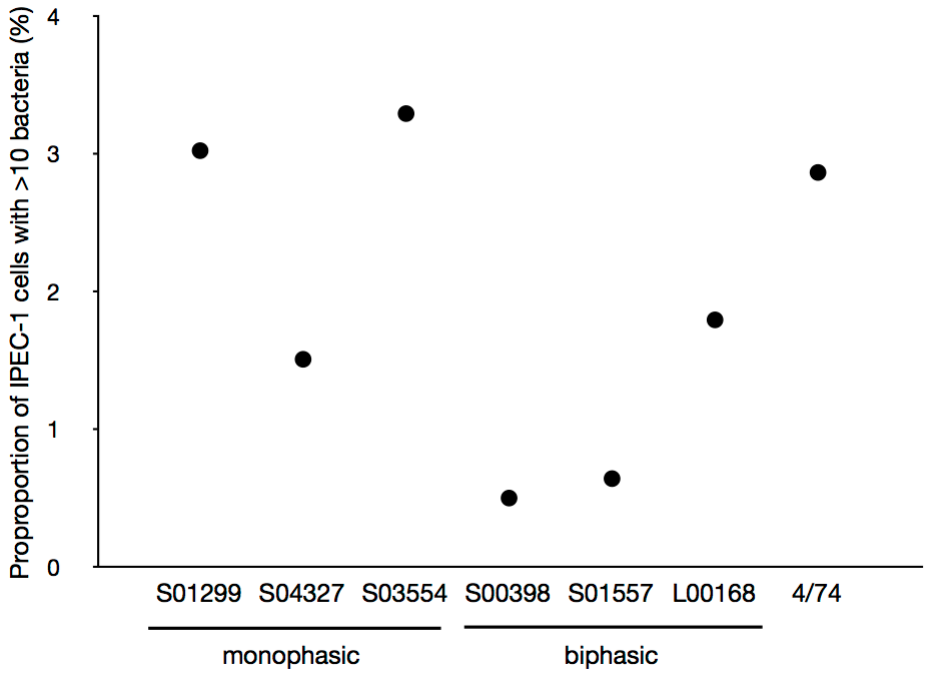
Figure 3.2 Comparison of proportion of IPEC-1 cells with cell-associated *Salmonella*.

IPEC-1 cells grown on glass coverslips were infected with *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and *S.* Typhimurium 4/74 at an MOI of 10 for 30 min. Following immunostaining of cell-associated bacteria, coverslips were analysed using a Nikon Eclipse E80i fluorescence microscope with oil-immersion lens (100x). Ten randomly selected fields were analysed per coverslip, amounting to at least 200 IPEC-1 cells per sample. Results are mean ± SEM.

A.


S01299
S04327
S03554
S00398
S01557
L00168
4/74
Proportion of IPEC-1 cells (%)
0
2
4
6
8
1
2
3
4
5
6-10
>10
Number of bacteria per IPEC-1 cell

B.


Proproportion of IPEC-1 cells with >10 bacteria (%)
0
1
2
3
4
S01299
S04327
S03554
S00398
S01557
L00168
4/74
monophasic
biphasic

C.

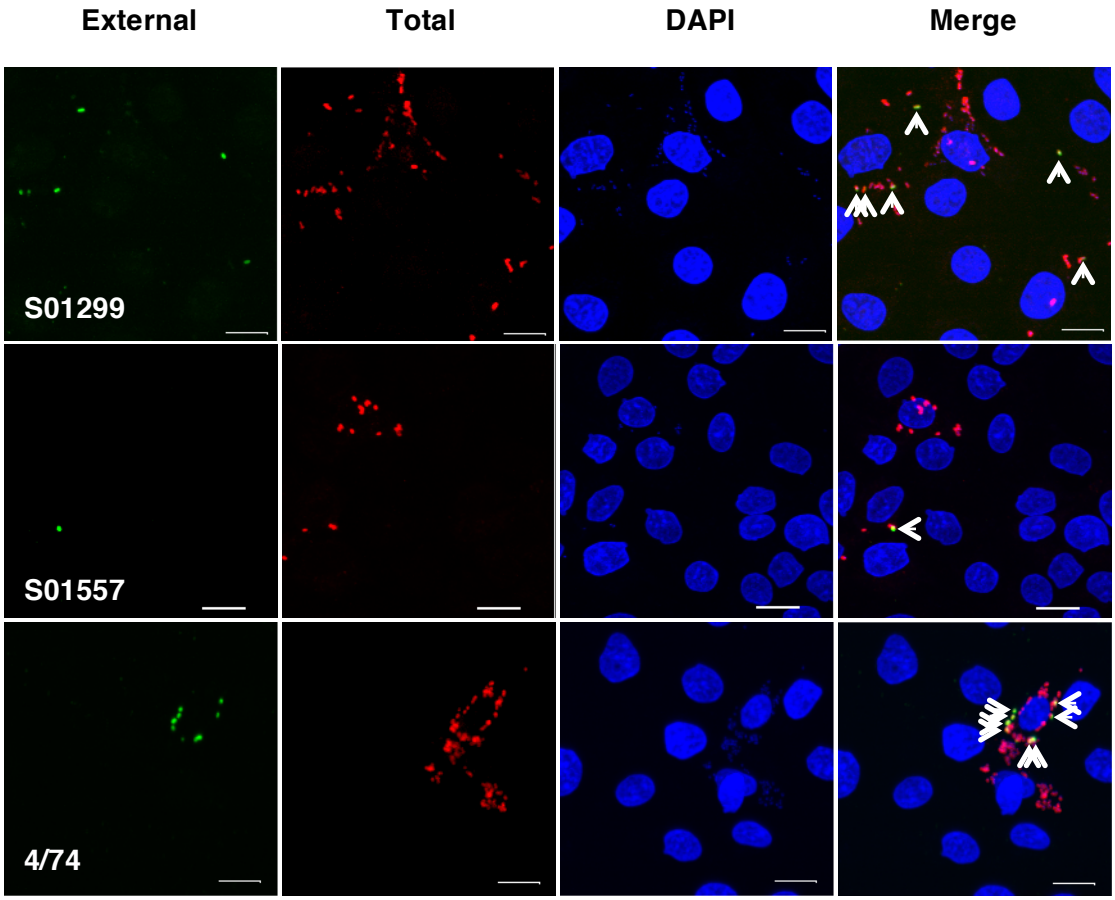

External
Total
DAPI
Merge
S01299
S01557
4/74

Figure 3.3 Distribution of *Salmonella* across IPEC-1 monolayers during early infection.

IPEC-1 cells grown on glass coverslips were infected with *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and *S.* Typhimurium 4/74 at an MOI of 10 for 15 (■) or 30 (■) minutes. Immunolocation of external (green) and total (red) bacteria was assessed by fluorescence microscopy and where an IPEC-1 cell (nuclei, blue) was associated with bacteria, the number of bacteria was estimated. Cells were permeabilised with 0.3% (w/v) Triton-X100 in PBS for 20 min. **A.** Mean ± SEM proportion of cells with *x* number of associated (adhered and invaded) bacteria. **B.** Summary of mean proportion of cells with greater than 10 bacteria. Data were calculated from three independent experiments in which at least 200 IPEC-1 cells were assessed. **C.** Representative confocal images of S01299 (4,12:i:-), S01557 (phase variable) and 4/74. Maximum intensity projections of 3D data sets are shown, with optical slices acquired at 1 μm intervals. Scale bars represent 15 μm. External bacteria are indicated with arrows.

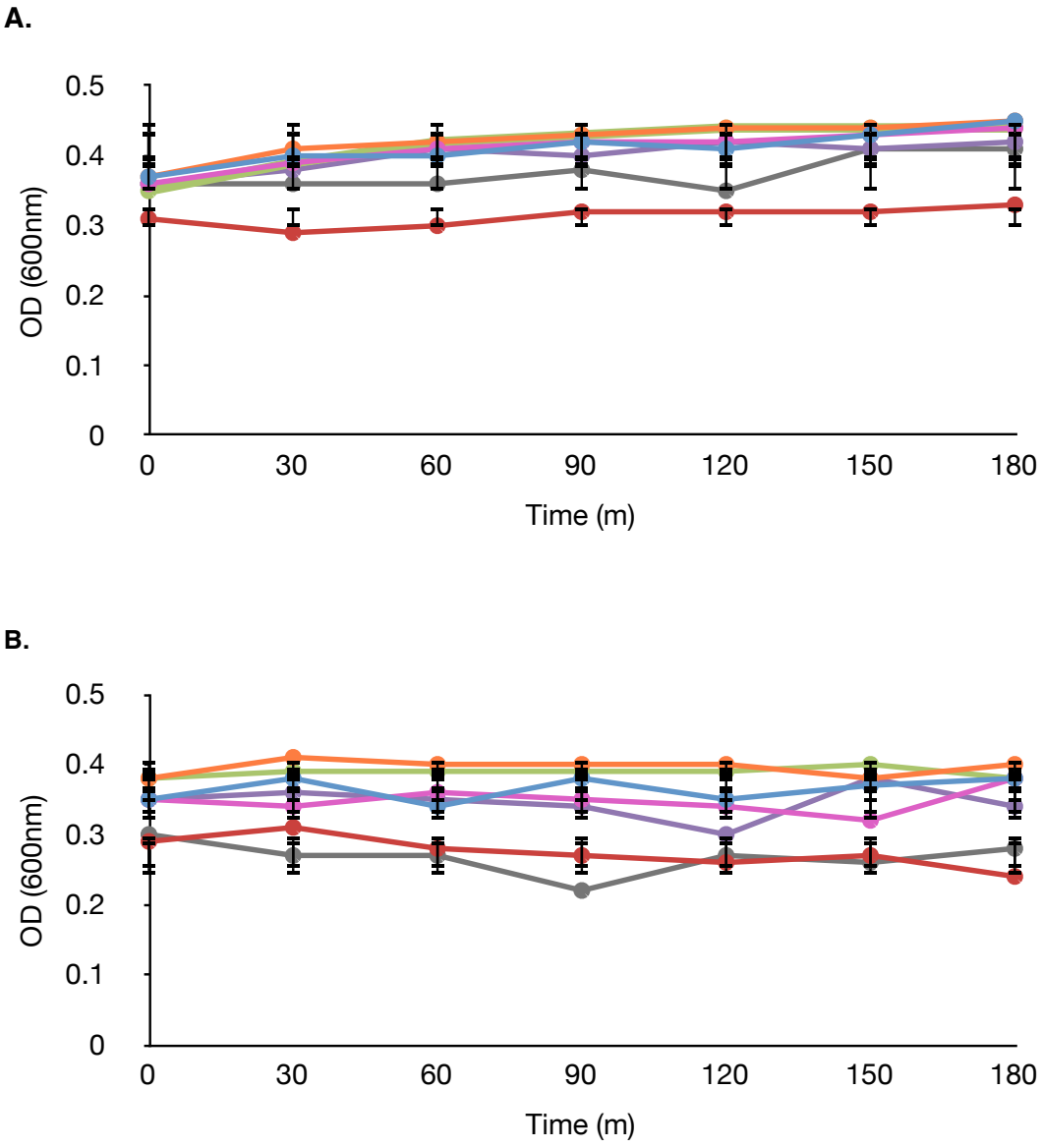


Figure 3.4 Assessment of autoaggregation behaviour of Salmonella isolates.

Salmonella 4,[5],12:i:- isolates S01299 (●), S04327 (●), S03554 (●) and Typhimurium isolates S00398 (●), S01557 (●), 4/74 (●) were grown to late-logarithmic phase at 37°C with shaking at 150pm. Cultures were vortexed to distribute the bacteria within the liquid culture before being left to stand at room temperature (**A**) or 4°C (**B**) for 3 h. Samples were collected at 30 min intervals from 0.5 cm below the liquid-air surface for determination of OD_{600}. Results are mean values ± SD, where n = 3.

3.3.2 SPI-1 gene expression

Because of the involvement of the SPI-1 T3SS and its associated effector proteins in *Salmonella* invasion into epithelial cells, the expression of this pathogenicity island by the isolates was quantified. Expression of the genes encoding the T3SS-1 needle apparatus are driven by the *prgH* promoter (Klein *et al.*, 2000) and expression of GFP under the control of this promoter has been used extensively as a reporter of SPI-1 expression (Clark *et al.*, 2011; Hautefort *et al.*, 2003; Humphrey *et al.*, 2011; Ibarra *et al.*, 2010; Perrett *et al.*, 2009). Analysis by repeated-measures ANOVA revealed that the expression of *prgH* over time was not significantly different between the different isolates (P = 0.707; Figure 3.5). Interestingly, *prgH* expression in stationary phase cultures (t = 0) was high (34-50%). This result conflicts with the findings of Humphrey *et al*. (2011), who found that just 7.8% of an overnight population of *S*. Typhimurium SL1344 were positive for *prgH*-driven GFP expression. One hour after the overnight cultures were subcultured into fresh LB medium, expression of *prgH* was low, with just 5.4% of the bacterial population positively expressing GFP. Expression levels remained low for the next 2 hours, after which they increased significantly between 3 h and 4 h from 6% to 31% ($P <$ 0.0001) and again between 4 h and 5 h to 42% ($P < 0.05$). The increase in the proportion of the bacterial population expressing *prgH* between these time points correlates with the logarithmic phase of growth. Although *prgH* expression by the *S.* Typhimurium isolate S01557 population seemed to be lower than that by the other bacterial cultures after 4 h growth (Figure 3.5B), this difference was not significant and was no longer present by 5 h (Figure 3.5C). GFP expression levels peaked at 6 h (48.3%), but appeared to be levelling off as the bacterial populations entered stationary phase. These data are consistent with previous studies in which *prgH* was shown to be heterogeneously expressed within *S*. Typhimurium populations, with a peak in expression levels between late log phase and stationary phase of growth (Clark *et al.*, 2011; Hautefort *et al.*, 2003; Humphrey *et al.*, 2011; Perrett *et al.*, 2009).

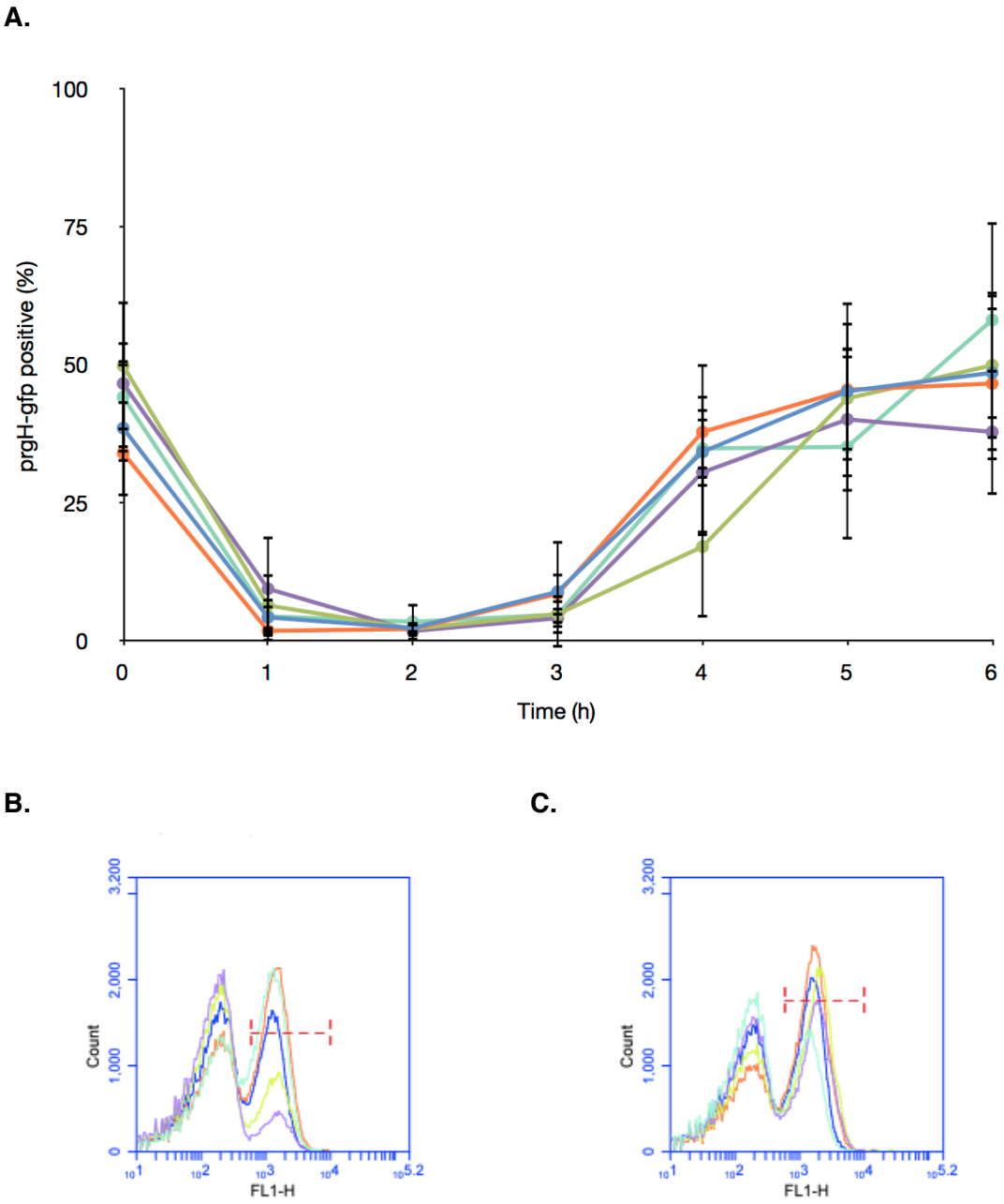


Figure 3.5 Quantification of SPI-1 expression, driven by the *prgH* gene.

Expression of GFP from the P*prgH-gfp* reporter plasmid was monitored over 6 h in *Salmonella* 4,[5],12:i:- isolates S01299 (GC004, ●) and S03554 (GC005, ●) and Typhimurium isolates S01557 (GC006, ●), L00168 (GC007, ●) and SL1344 (SH001, ●) from stationary phase (t = 0) through logarithmic phase (t = 1-6) of growth. Samples were fixed and analysed by flow cytometry to determine the proportion of the population positively expressing GFP (**A**). Results are mean ± SD, where n = 3. Representative flow cytometry data acquisition outputs showing fluorescence intensity and the number of detection events in the FL1 channel after 4 h (**B**) and 5 h (**C**) growth, where the red marker indicates the *gfp*$^+$ proportion of the population.

3.3.3 Invasion into non-porcine cells

3.3.3.1 Caco-2 cells

Although the focus of this work is on the infection biology of pig-associated *Salmonella* field isolates in a porcine model of infection, the fact that both monophasic and biphasic DT193 isolates have been implicated in human infection meant that quantification of invasion into human intestinal epithelial cells was both relevant and important. The number of intracellular bacteria within Caco-2 cells was determined by colistin-protection assay 1 h post-infection. The proportion of the bacterial inoculum that successfully invaded was subsequently calculated and the results were analysed using the Kruskal-Wallis non-parametric test due to the spreads of data being unequal. The Kruskal-Wallis test ranks responses from all groups together and then applies a one-way ANOVA to the ranks rather than to the original observations. A significant *P* value indicates that the null hypothesis, that all of the groups have the same median, should be rejected. Determination of the MIC of colistin for each isolate confirmed that the concentrations of the antibiotic used in the protection assays were sufficiently high to kill the extracellular bacteria. The MIC for all of the isolates was 16 mg/l (Figure 3.6).

The Caco-2 invasion results are illustrated in Figure 3.7. Statistical analysis revealed that there was at least one difference between the isolates of *Salmonella* in invasion levels ($P < 0.05$). Median levels of invasion were lowest for 4,12:i:- isolate S01299 and Typhimurium isolate L00168. The median proportion of inoculated bacteria that successfully invaded the Caco-2 cells ranged from 0.03 to 0.15%. These data are consistent with previous studies in which similar low levels of invasion were seen following infection of Caco-2 cells with *S*. Typhimurium (Kim & Wei, 2007; Sierro *et al*., 2001; Sirsat *et al*., 2011) and in a human intestinal *in vitro* organ culture assay for infection (Haque *et al*., 2004). However, other studies have reported higher levels of *S.* Typhimurium invasion into Caco-2 cells, with invasion frequencies ranging from 4-38% after 1 h infection (Gagnon *et al*., 2013; Hölzer & Hensel, 2012; Karatzas *et al*., 2007; Mills & Finlay, 1994; Salisbury *et al*., 2011).

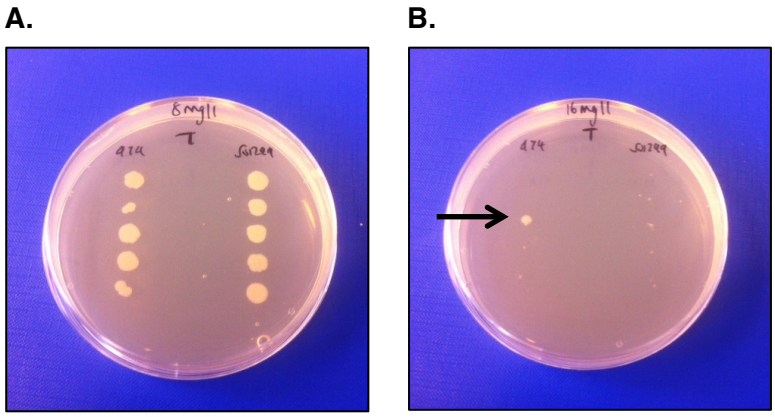


Figure 3.6 Minimum inhibitory concentration of colistin.

Spots of *Salmonella* 4,[5],12:i:- and Typhimurium isolates containing 1 x 10^5 CFU per spot were dispensed onto nutrient agar plates containing doubling dilutions of colistin sulphate. After 24 h incubation at 37°C the plates were analysed to determine the minimum concentration of colistin that inhibited colony growth. A concentration of 8 mg/l was insufficient to inhibit growth (**A**). Growth of all isolates, except one (indicated with a black arrow) that was not included in the final panel of isolates for this study, was inhibited at 16 mg/l (**B**).

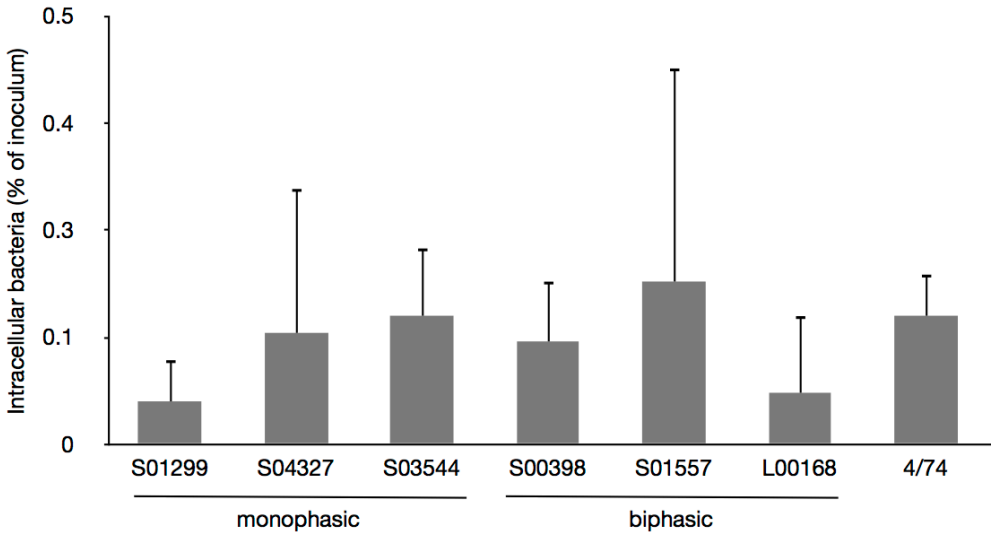


Figure 3.7 Proportion of *Salmonella* inoculum that invaded Caco-2 cells 1 h post-infection.

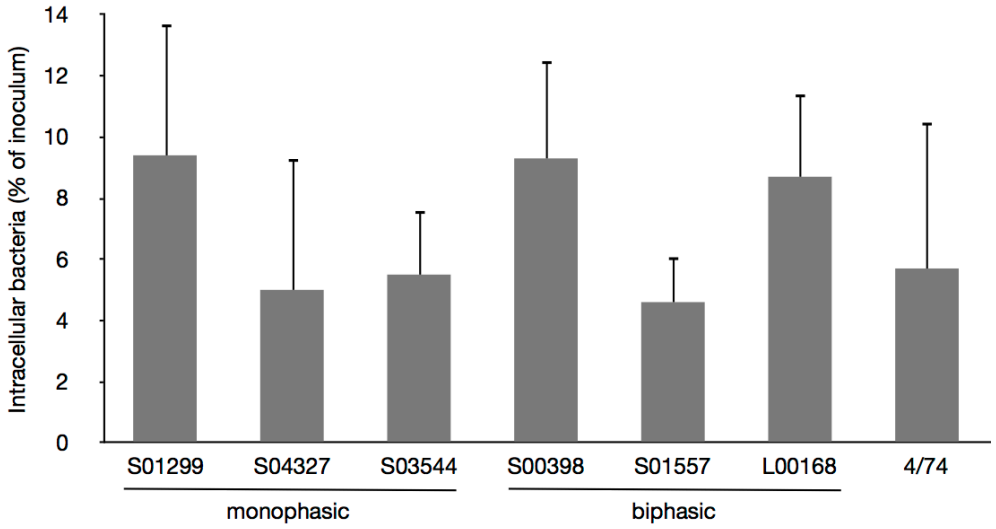
Monolayers of Caco-2 cells were infected with late-log phase *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and *S.* Typhimurium 4/74. After 1 h incubation, extracellular bacteria were killed with colistin, cells lysed and intracellular bacteria enumerated by plating serial dilutions onto nutrient agar plates. Results are mean ± SD of three independent experiments performed in triplicate.

3.3.3.2 RAW264.7 macrophages

In addition to the active invasion of non-phagocytic cells by *Salmonella*, bacterial uptake by phagocytic cells, including macrophages and dendritic cells, is an important stage of infection following breach of the intestinal epithelium. These cells represent primary sites of *S*. Typhimurium survival and replication, which both facilitate the establishment of systemic infection (Fields *et al.*, 1986). Determination of the uptake and intracellular replication of *S*. Typhimurium in RAW264.7 macrophage-like cells is a commonly used method for the assessment of bacterial virulence potential and intracellular phenotype. Results from the colistin-protection assays revealed some variation between the isolates in the proportion of inoculated bacteria located within the RAW264.7 cells after 30 min (Figure 3.8A). Once again, the data were analysed using the Kruskal-Wallis rank test, which identified that the median percentages of intracellular bacteria were not equal among the different isolates ($P < 0.05$). Specifically, 4,12:i:- isolate S01299 and biphasic isolate L00168 had the highest ranking medians, with 11.1% and 10.1% of inoculated bacteria becoming internalised, respectively. Monophasic (4,5,12:i:-) isolate S04327 and biphasic isolate S01557 had the lowest ranking medians.

The rate of intracellular *Salmonella* replication was calculated as the ratio of intracellular bacteria at 30 min to at 24 h (Figure 3.8B). All isolates demonstrated an increase in bacterial numbers between these time points, indicating survival and/or intracellular proliferation. However, the level of replication was less than 1-fold and the variation in intracellular bacterial numbers for each isolate was quite wide. Statistical analysis revealed that the median increase in intracellular bacteria over 24 h was not significantly different between the isolates ($P = 0.115$).

A.



B.

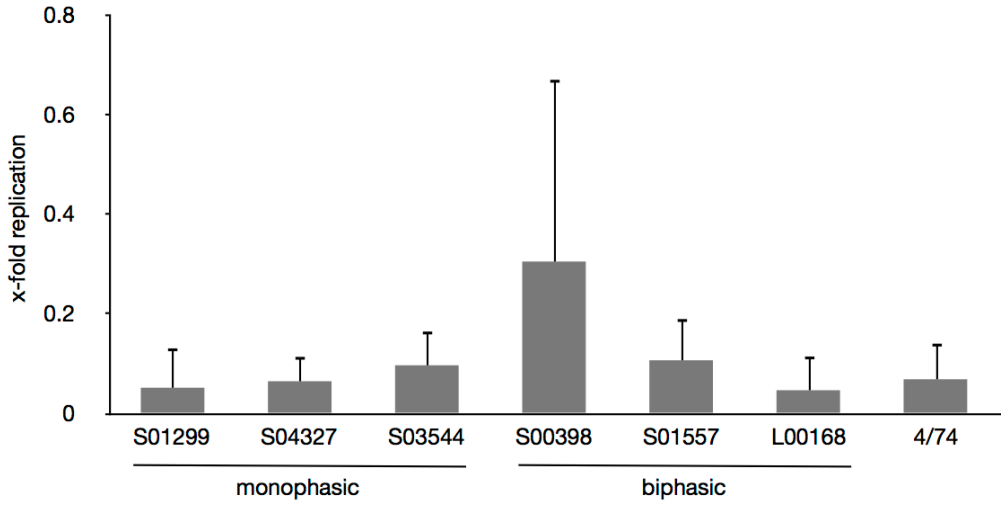


Figure 3.8 Interactions between Salmonella isolates and RAW264.7 cells.

Monolayers of RAW264.7 cells were infected with stationary phase *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and *S.* Typhimurium 4/74 at an MOI of 10. After the indicated infection time, extracellular bacteria were killed with colistin, cells lysed and intracellular bacteria enumerated by plating serial dilutions onto nutrient agar plates. **A.** Proportion of inoculated bacteria that were intracellular after 30 min. **B.** The number of intracellular bacteria after 24 h incubation was divided by the 30 min values to calculate the x-fold intracellular replication. Results are mean ± SD of three independent experiments, where n = 3.

3.4 Discussion

This study investigated the virulence of monophasic and biphasic *Salmonella* phage type DT193 strains isolated from pig faeces on British farms in a porcine intestinal epithelial cell culture model of early intestinal infection. The rapid and worldwide spread of phage type DT193 shows similarities to the global epidemic of *S.* Typhimurium DT104 that occurred during the 1990s. While it was originally thought that infection with DT104 resulted in more severe clinical outcomes than infection with other *Salmonella* phage types (Wall *et al.*, 1994), a number of studies subsequently showed that DT104 isolates do not exhibit an enhanced capacity for invasion of epithelial cells or for replication within macrophages *in vitro* (Adaska *et al.*, 2008; Allen *et al.*, 2001; Bergeron *et al.*, 2009; Carlson *et al.*, 2000). Results presented here suggest that the same can be said for DT193 isolates.

Although invasion and intracellular proliferation are often used as a gauge of virulence and infection potential, both represent single stages in a complex interplay between *Salmonella*, the host and the environment. Indeed, the virulence of these strains and their rapid dissemination may be the result of other aspects of their phenotype. For example, monophasic and biphasic variants of *Salmonella* phage type DT193 have both been shown to survive longer in pig faeces than other serotypes, which may facilitate faecal-oral transmission and therefore contribute to their increased prevalence (Rajtak *et al.*, 2012). Overall, the adhesion and invasion results suggest that most *S.* Typhimurium DT193 and 4,[5],12:i:- variants may be capable of colonising the small intestine of pigs and causing enteritis. This is because all isolates, except S00398 and S01557, adhered to and invaded IPEC-1 cells to a similar level as the control strain 4/74, which is known to invade porcine ileal mucosa to a high level *in vivo* (Bolton et al., 1999; Paulin et al., 2007). Indeed, previous work has shown that the same DT193 isolates used in this study are capable of colonising the caeca of chickens (Parsons *et al.*, 2013). However, *in vivo* experiments would need to be conducted to confirm this capability in the pig host.

The role of flagella during interactions with intestinal epithelial cells is multi-faceted. Not only is flagella-based motility fundamental to the occurrence of the *Salmonella* swimming dynamics on host-cell surfaces that precede invasion (Misselwitz *et al.*, 2012), but the flagella of *Salmonella* and other bacterial species have also been implicated in mediating adhesion to host cells (Elvidge, 2013; Lillehoj *et al.*, 2002;

Roy *et al.*, 2009; Tasteyre *et al.*, 2001). Results presented here show that monophasic expression of the phase 1 flagellar antigen, FliC, does not affect the ability of 4,[5],12:i:- isolates to adhere to and invade IPEC-1 cells. The levels of adhesion and invasion of the 4,[5],12:i:- isolates were not significantly different to that of the DT193 isolates expressing both phases of flagellar antigen. Although an explanation for why *Salmonella* bacteria exhibit flagellar phase variation has not yet been determined, preliminary work has shown that the bacteria may be able to "sense" the restriction of one flagella phase by circulating antibodies and consequently switch expression to the other (Elvidge, 2013). A study involving phase-locked derivatives of *S*. Typhimurium SL3201, expressing FliC or FljB only, showed no differences from the wild-type parent strain in adherence and invasion of mouse epithelial cells or in a bovine ligated loop assay (Ikeda *et al.*, 2001). The authors concluded that flagellar phase variation is not involved in the intestinal stage of infection. The results presented here are consistent with this theory.

However, another study investigating the binding and invasion of the jejunum-derived porcine epithelial cell line IPEC-J2 by *S*. Typhimurium SL1344 single mutant strains (Δ*fliC* and Δ*fljB*) observed that both mutants showed reduced binding and invasion compared to the wild-type strain. An explanation for the differences in results could be the polarisation state of the different cell lines used; IPEC-J2 cells are polarised, whereas IPEC-1 cells are not. Polarised intestinal epithelial cells form distinct apical and basolateral membranes and microvilli, characteristics that more closely resemble the epithelial cells lining the intestine *in vivo* (Criss et al., 2001; Finlay et al., 1988; McCormick, 2003). Boyen *et al.* (2009) reported that *S.* Typhimurium invasion into IPEC-J2 was markedly increased compared with invasion into the non-polarised cell line IPI-2I. Further comparisons between *Salmonella* invasion into polarised and non-polarised cells have found that different Rho GTPases, mediators of actin rearrangements, are required for bacterial entry into the different cell types. Chen *et al*. (1996a) identified that *Salmonella* invasion into non-polarised HeLa cells was primarily dependent on activation of the CDC42 Rho GTPase, while Criss *et al*. (2001) found instead that activation of RAC1 and not CDC42 was required for apical bacterial entry into polarised MDCK cells. The situation is further complicated by the fact that the *sopE* gene is not homogenously present in the panel of isolates used in this study. As described in Chapter 2, SopE is a GEF that activates both CDC42 and RAC1 and while no consistent relationship

between *sopE* presence and invasiveness into cultured cells has been identified, SopE has been correlated with increased membrane ruffle size and speed of induction of actin rearrangements (Clark *et al.*, 2011). Use of a polarised cell line and *sopE*$^{+}$ *S*. Typhimurium SL1344 by Elvidge (2013), compared with use of a non-polarised cell line and a mixture of *sopE*$^{+}$ and *sopE*$^{-}$ strains in this study may explain the differences in results. For example, none of the phase variable DT193 isolates in this study possessed *sopE*. Instead, comparison of the *sopE*$^{+}$ monophasic isolates with *sopE*$^{+}$ biphasic isolates would have made for a better-controlled study and may have revealed similar differences to Elvidge (2013) in invasive phenotype between the two groups.

Given the short infection times studied here, it is believed that the observed foci of infection, with high numbers of bacteria interacting with single host cells, were unlikely to be due to hyperreplication of cytosolic bacteria, a phenomenon described previously by Knodler et al. (2010). Preferential invasion of specific target cells, namely mitotic cells and cells with membrane ruffles, and cooperative invasion at these target sites, has been observed by others during *Salmonella* infection of HeLa cells (Misselwitz *et al.*, 2010; Misselwitz *et al.*, 2011a; Misselwitz *et al.*, 2012). However, without data pertaining to the morphology of the IPEC-1 cells experiencing high bacterial loads in this study, it is not possible to determine whether this was targeted cell invasion. Similarly, the reason why some of the isolates displayed greater incidence of "hyperinvasion" than others remains elusive.

Infection of IPEC-1 cells with the *Salmonella* isolates for a longer time may have revealed more about the intracellular adaptation of the monophasic and biphasic DT193 isolates. Hautefort *et al*. (2008) found that both flagellin genes, *fliC* and *fljB*, were upregulated by *Salmonella* released from epithelial cells 6 h post-infection, but interestingly, *fliC* was upregulated to double the level of *fljB*. The authors suggested that *de novo* production of flagella by *S*. Typhimurium inside epithelial cells primes the bacteria for motility upon their release following death of the host cell, a theory explored by others (Knodler *et al.*, 2010). However, they did not comment on the differential expression of *fliC* and *fljB*. For whatever reason, it could be that FliC flagellin is preferentially expressed by intra-epithelial *Salmonella* over FljB. It would therefore be interesting to investigate whether the complete lack of FljB expression

by the 4,[5],12:i:- isolates would have any effect on survival and replication within IPEC-1 cells over an extended time period.

Expression of SPI-1 genes has been shown to play a crucial role in the invasion and colonisation of the porcine gut (Boyen *et al.*, 2006c). Although differences in the invasiveness of the DT193 isolates were not statistically significant, some variation between the isolates was evident. Except perhaps in the case of flagellar phase variable isolate S01557, the observed variation in invasion was not a result of disparity in SPI-1 expression, as shown by the P*prgH-gfp* results. The reduced invasion of S01557, however, could be linked to its reduced *prgH* expression during the logarithmic phase of growth. Reduced *prgH* expression by *S.* Typhimurium populations has previously been shown to correlate with reduced invasiveness into cultured epithelial cells (Clark *et al.*, 2009; Clark *et al.*, 2011). All of the invasion assays were performed by inoculating cells with bacterial subcultures grown for 3.5 h. Again, although the difference was not statistically significant, S01557 clearly expressed *prgH* in a lower proportion of the population compared to the other isolates 3-4 hours after the overnight population was subcultured into fresh LB broth. Overall, it would be prudent to conclude that strain S01557 is likely to be attenuated for virulence *in vivo* given its reduced motility (Chapter 2), reduced *prgH* expression and consequent reduced invasive ability.

As a model of intestinal infection in humans, invasion of the DT193 isolates into Caco-2 cells was assessed. The results from this assay are mostly inconclusive but it is worth noting that there was no obvious difference between the monophasic and biphasic isolates in their ability to invade. As with the porcine intestinal epithelial cells, polarisation of the Caco-2 cells by growing them on permeable supports may have provided a more optimal model of infection.

Porcine monocytes respond *in vitro* to *S*. Typhimurium with phagocytosis, oxidative burst and bacterial killing (Boyen *et al.*, 2008b; Donné *et al.*, 2005; Riber & Lind, 1999) In the absence of appropriate porcine macrophage cell lines and with insufficient time to develop primary culture methods, the well-characterised murine monocytic macrophage cell line RAW264.7 was selected to model *Salmonella* DT193 replication within mononuclear cells, which would be likely to occur in the GALT. Invasion into the RAW264.7 cells was much greater than into IPEC-1 and Caco-2 cells, which is unsurprising given that *Salmonella* is phagocytosed by

macrophages as well as taken up via active invasion. A similar level of macrophage invasion as reported here was seen in another study of *S*. Typhimurium internalisation into RAW264.7 macrophages (Monack *et al*., 1996) but the results are not directly comparable because of differences in MOI and infection time. A lower level of internalisation by *S*. Typhimurium into these cells was observed in another study, in which just 1% of the inoculated bacteria were internalised during 30 min incubation. However, the authors used a lower MOI and did not centrifuge the bacteria onto the cells (Hölzer & Hensel, 2012). These authors infected RAW264.7 macrophages with bacteria grown to stationary phase, a growth condition that induces a non-invasive phenotype (Lee & Falkow, 1990). Therefore, their results correspond only to the phagocytosis-mediated uptake of *Salmonella* as opposed to the SPI-1-mediated active invasion by induction of membrane ruffling and macropinocytosis. Although infection of RAW264.7 cells in this study was also with bacteria grown to stationary phase, results from the quantification of P*prgH-gfp* expression (Figure 3.5) suggest that these bacteria were invasive, which may explain the higher rates of internalisation compared to Hölzer and Hensel (2012). The expression of SPI-1 by the stationary phase cultures could have been a result of low oxygen availability during overnight culture, a condition that is known to stimulate expression of this pathogenicity island (Bajaj *et al*., 1996; Ernst *et al*., 1990; Lee & Falkow, 1990).

Salmonella Typhimurium has been shown to persist in high numbers within porcine alveolar macrophages, despite this serotype not typically being associated with systemic disease in pigs (Watson *et al*., 2000). Therefore, the persistence of the *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates within macrophages was investigated. Quantification of *S*. Typhimurium replication in RAW264.7 macrophages by other studies showed much greater levels than observed in this study, with an increase in the number of intracellular bacteria between time points of up to 100-fold (Govoni *et al*., 1999; Hölzer & Hensel, 2012; Lim *et al*., 2006; Monack *et al*., 1996; Sha *et al*., 2004). Monack *et al*. (1996; 2001) demonstrated that late-log phase, invasive *S*. Typhimurium SL1344 induces caspase-1-dependent apoptosis in RAW264.7 macrophages via a specific pathway associated with the membrane ruffling mechanism of active invasion. They also showed that *S.* Typhimurium SL1344 grown to stationary phase, which are consequently phenotypically non-invasive, were unable to induce membrane ruffling and

apoptosis. Since the stationary phase bacteria in this study appeared to still exhibit an invasive phenotype, as shown by the P*prgH-gfp* results, the low levels of intracellular replication observed in RAW264.7 macrophages may be a result of invasion-induced macrophage apoptosis. This cytotoxicity would have caused release of intracellular bacteria into the culture medium and subsequent bacterial death, thus limiting the levels of bacterial recovery after 24 h. SPI-1-dependent induction of early cytotoxicity by *S*. Typhimurium has also been demonstrated in porcine pulmonary alveolar macrophages (Boyen *et al.*, 2006a).

It is important to interpret the results of the assays with RAW264.7 cells with caution, given that the degree of *Salmonella* intracellular growth and survival is affected by the source of isolation (blood, lungs, spleen, bone marrow or peritoneum) of the macrophages in both murine and porcine models (Boyen *et al.*, 2009; Buchmeier & Heffron, 1989; Hensel *et al.*, 1998). Additionally, it has been shown that porcine immune cells do not upregulate inducible nitric oxide synthase (iNOS) following stimulation (Akunda *et al.*, 2001; Donné *et al.*, 2005; Pampusch *et al.*, 1998), suggesting that production of reactive nitrogen species (RNS) by iNOS is not an important feature of the innate immune response in pigs. In mice, the enzymes iNOS and phagocyte oxidase (phox) in macrophages display bactericidal activity by catalysing the synthesis of RNS and reactive oxygen species (reviewed by Vazquez-Torres and Fang, 2001b). A deficiency in either phox or iNOS attenuates the ability of macrophages to control *Salmonella* replication and phox and iNOS-deficient mice succumb to *Salmonella* infection due to an inability to control bacterial replication (Mastroeni *et al.*, 2000; Shiloh *et al.*, 1999; Vázquez-Torres *et al.*, 2000a). Therefore, the production of iNOS by the RAW264.7 cells may have limited the intracellular replication of the *Salmonella* isolates in this study in a way that is not relevant to infection in pigs.

Taken together, the data presented in this chapter show that isolates of *Salmonella* 4,[5],12:i:- and Typhimurium belonging to phage type DT193 and originating from pigs are capable of adhering to and invading porcine intestinal epithelial cells *in vitro*. Confirmation with *in vivo* experiments is necessary, but the data suggest that these isolates would be capable of colonising the intestinal mucosa, an attribute that would explain their presence in European pig herds. The isolates were also invasive into human intestinal epithelial cells and murine monocytic macrophages but further development of these models is required to obtain more relevant results.

Finally, no clear-cut differences between monophasic and biphasic isolates were identified, suggesting that flagellar phase variation is not involved in early infection of the porcine gut mucosa.

4 Host Immune Response During Early Infection

4.1 Introduction

Over and above their primary function as a physical barrier, intestinal epithelial cells exhibit a number of immunologically important mechanisms, which directly or indirectly limit infection by invasive pathogens (reviewed by Oswald, 2006). One of these mechanisms is the secretion of pro-inflammatory chemokines and cytokines, following the stimulation of TLRs by pathogens, which leads to the initiation of both innate and adaptive immune responses. Failure by intestinal epithelial cells to respond appropriately to antigenic stimuli can result in chronic inflammation and impaired digestive function.

Recognition of PAMPs by transmembrane TLRs of intestinal epithelial cells triggers the innate immune response via activation of signal transduction cascades such as the NF-κB and MAPK pathways. An important PAMP of *Salmonella* and other Gram-negative bacteria is flagellin, the principal component of flagellar filament. Flagellin induces a pro-inflammatory transcriptional response culminating in the release of chemokines and cytokines, such as IL-8, from intestinal epithelial cells by acting as a stimulatory ligand for TLR-5 (Hayashi *et al.*, 2001; Zeng *et al.*, 2003; Zeng *et al.*, 2006). TLR-5 is only able to recognise flagellin in its monomeric form, not as a filamentous structure (Smith *et al.*, 2003). Flagellin monomers present in the lumen of the intestine as a result of leakage during bacterial flagellar assembly (Komoriya *et al.*, 1999) and/or physical shearing from the bacterial cell are internalised by intestinal epithelial cells via a TLR-5-dependent mechanism (Eaves-Pyles *et al.*, 2011). Following internalisation, flagellin monomers bind to basolateral TLR-5 receptors and stimulate secretion of IL-8 chemokine. The primary role of IL-8 is as a chemoattractant for recruitment of neutrophils to the lamina propria (McCormick *et al.*, 1995), an event that is instrumental in causing both the pathology of gastroenteritis and clearance of bacteria from the gut (Zhang *et al.*, 2003b).

The flagella filaments of *S.* Typhimurium are composed of one of two flagella monomers, FliC or FljB, which can be alternately expressed by phase variation (Aldridge *et al.*, 2006; Bonifield & Hughes, 2003). Although FljB and FliC are almost entirely genetically identical, amino acids exposed on the surface are divergent, resulting in different antigenicities. Therefore, it has been postulated that phase

variation of these proteins enables *S.* Typhimurium to elude host cellular immune recognition (Aldridge *et al.*, 2006; Anon., 2012a; Elvidge, 2013; Ikeda *et al.*, 2001). Indeed, one study observed that most $CD4^+$ T lymphocytes responding to a *Salmonella* infection were directed at flagellin epitopes (Bergman *et al.*, 2005). Thus, if a host has acquired immunity against one of the two flagellin types, *Salmonella* cells that are able to change their flagellar antigen expression should theoretically have a selective advantage. However, the true biological significance of flagellar phase variation has not yet been determined.

In addition to activation of pro-inflammatory responses, TLR-5 recognition of flagellin also initiates both apoptotic and anti-apoptotic signaling in intestinal epithelial cells (Paesold *et al.*, 2002; Vijay-Kumar *et al.*, 2006; Zeng *et al.*, 2006). Zeng and colleagues (2006) identified that in addition to transcriptional upregulation of pro-inflammatory genes, intestinal epithelial cells also activate a panel of anti-apoptotic mediator genes such as cIAP-1, cIAP-2 and A20, in response to flagellin. These genes are able to inhibit caspase activation and subsequent pro-apoptotic signaling (Deveraux & Reed, 1999; Karin & Lin, 2002) and are induced in a NF-κB-dependent manner (Burstein & Duckett, 2003). The authors also found that caspases of the extrinsic pathway (caspase 8) and intrinsic pathway (caspase 9) were activated in polarized human intestinal epithelial cells (T84) following stimulation with purified flagellin and live wild-type *S.* Typhimurium. This apoptotic stimulation was markedly enhanced when pro-inflammatory and anti-apoptotic NF-κB pathways were blocked.

It is presumed that flagellin-dependent initiation of programmed cell death evolved as a primitive immune mechanism by which the host organism can eliminate infected cells without fatal effects to the animal (Ameisen, 2002; James & Green, 2002). Furthermore, pro-inflammatory upregulation of anti-apoptotic signaling in parallel to pro-apoptotic signaling is thought to have developed as a means of preventing unimpeded irreversible cell damage and preserving mucosal defense (Zeng *et al.*, 2006). Indeed, Vijay-Kumar *et al.* (2006) showed in a murine model of salmonellosis that infection with aflagellate bacteria, which are consequentially more potent activators of epithelial caspases, resulted in a delayed but markedly exacerbated mucosal inflammation and elevated extraintestinal and systemic bacterial load. Therefore, the balance between pro-inflammatory and pro-apoptotic signaling is a delicate one that can dramatically influence pathogenesis of the infection. Given the integral role of flagellin in driving host-pathogen infection

dynamics during *Salmonella* infection, the ability of 4,[5],12:i:- and Typhimurium DT193 isolates to stimulate inflammatory and apoptotic responses in porcine intestinal epithelial cells was evaluated.

4.2 Materials and Methods

4.2.1 *Salmonella* isolates

The isolates of bacteria used throughout this study are detailed in Chapter 2 (Table 2.1 and 2.4) and Chapter 3 (Table 3.1). The isolates of pig origin were kindly donated by the Animal Health Veterinary Laboratories Agency (Surrey, UK) who isolated them from pig faeces on farms in Great Britain.

4.2.2 Bacterial culture conditions

All *Salmonella* isolates were stored on Microbank™ cryoprotective beads (Pro-Lab Diagnostics Inc., UK) at -80°C. Using aseptic technique, isolates were streaked from frozen stocks onto nutrient agar and incubated aerobically for 24 h at 37°C. Stationary phase cultures were prepared by inoculating 10 ml standard Miller formulation Luria-Bertani (LB) broth with 2-3 colonies from a nutrient agar plate using a sterile loop. Cultures were incubated overnight for 16-18 h at 37°C, 150 rpm in an orbital shaker. Late-logarithmic phase cultures were achieved by diluting stationary phase cultures 1:100 (v/v) into fresh LB broth followed by incubation at 37°C, 150 rpm for a further 3.5 hours.

4.2.3 Cell culture

4.2.3.1 IPEC-1

The IPEC-1 cell line is an undifferentiated intestinal epithelial line derived from the small intestine of a neonatal unsuckled piglet (Gonzalez-Vallina *et al.*, 1996). This line was a kind gift from Dr. Tristan Cogan, University of Bristol. Cells were maintained in 75 cm^2 plastic cell culture flasks (Cellstar, Greiner Bio-One Ltd., UK) at 37°C in a humidified atmosphere of 5% CO_2. They were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Life Technologies Ltd., UK) supplemented with 5% foetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium (ITS Premix; BD

Biosciences, UK) and 0.5 ng/ml human epidermal growth factor, hereafter referred to as IPEC-1 medium. Continuous cultures of IPEC-1 cells were maintained by passaging at a 1:3 or 1:5 ratio. Subculture was performed as described in Chapter 3 (Section 3.2.3.1).

4.2.4 Determination of IL-8 and TLR-5 gene expression by real-time quantitative PCR

4.2.4.1 Infection of IPEC-1 cells

For determination of the innate immune response of porcine intestinal epithelial cells to invading *Salmonella*, IPEC-1 cells were first seeded into 24-well tissue culture plates and incubated at 37°C, 5% CO_2 in antibiotic-free IPEC-1 medium for 24 h to give confluent monolayers with a final density of 1 x 10^6 cells per well. Late-log phase cultures of *Salmonella* were added to each well to achieve an approximate MOI of 10 bacteria per IPEC-1 cell. An equivalent volume (50 µl) of sterile LB broth was added to three wells per plate to serve as uninfected control samples. Assay plates were incubated for 1 hour at 37°C in air, after which time the medium was aspirated and 350 µl lysis Buffer RLT (Qiagen Ltd., UK) containing 1% (v/v) β-mercaptoethanol added to each well. Five minutes later the cell monolayers were disrupted by agitation with a pipette tip and the cell homogenates were collected into microcentrifuge tubes for storage at -80°C. Assays were performed in triplicate and samples were collected from 3 independent experiments.

4.2.4.2 Isolation of total RNA

Total ribonucleic acid (RNA) was isolated from the cell homogenates using an RNeasy Mini Kit (Qiagen Ltd., UK) according to the manufacturer's instructions. Briefly, 70% (v/v) ethanol was added to each sample in a 1:1 ratio, mixed well by pipetting and 700 µl of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube. After centrifugation for 15 s at 8000 x *g* the flow-through was discarded and additional centrifugation steps were performed with buffers supplied in the kit. Finally, RNA was eluted from the spin column membrane by addition of 50 µl RNase-free water and centrifugation for 1 min at 8000 x *g*. The concentration of total RNA isolated from each sample was determined using a NanoDrop spectrophotometer (Thermo Scientific, UK). Purified RNA samples were stored at -80°C until required.

4.2.4.3 qRT-PCR

RNA from sample and uninfected control IPEC-1 cells was subjected to one-step quantitative reverse transcription PCR (qRT-PCR) using a Rotor-Gene Probe RT-PCR kit (Qiagen Ltd., UK) on a Rotor-Gene Q (Qiagen Ltd., UK). Sequence-specific TaqMan Assay primer-probe sets for porcine IL-8 and TLR-5 and eukaryotic 18S (Life Technologies, UK) were included in the reaction tubes with 10 ng sample RNA. Reaction mixtures were setup using a QIAgility instrument, performed in triplicate and normalised to eukaryotic 18S ribosomal RNA. Each reaction tube contained 10 μl Rotor Gene MasterMix, 0.2 μl Rotor Gene reverse transcription enzyme, 1 μl TaqMan gene expression assay and 1 μl RNA. The following cycling conditions were used for amplification: 50°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The relative levels of IL-8 and TLR-5 gene expression were quantified using the $2^{-\Delta\Delta Ct}$ method. The threshold for C_T values was set for each gene and the average C_T value for each triplicate set of samples was calculated. C_T values were first normalised to the endogenous control and then to the uninfected control group. Expression levels were represented as the fold-change in expression compared to the uninfected control.

4.2.5 Determination of caspase activation by flow cytometry

4.2.5.1 Infection of IPEC-1 cells

IPEC-1 cells were seeded into 24-well tissue culture plates as described in Section 4.2.4.1. Late-log phase cultures of *Salmonella* were added to each well to achieve an approximate MOI of 10 bacteria per IPEC-1 cell. An equivalent volume (50 μl) of sterile LB broth was added to three wells per plate to serve as uninfected control samples. Assay plates were incubated for 2-8 h at 37°C, 5% CO_2.

4.2.5.2 Fluorogenic staining of active caspases

Activation of caspases in IPEC-1 cells was detected using the Apo Logix caspase detection kit (Cell Technology, Mountain View, CA), which uses a carboxyfluorescein (FAM) labelled peptide fluoromethyl ketone (FMK) caspase inhibitor, according to the manufacturer's protocol. The caspase inhibitor is cell permeable and non-cytotoxic. After the appropriate infection time, medium was aspirated from each well of the 24-well plate and IPEC-1 cells were released from

the plastic by addition of 500 µl trypsin-EDTA for 5 min. Trypsin activity was disabled through addition of 500 µl antibiotic-free IPEC-1 medium and released cells were collected into 12 x 75 mm^2 polystyrene tubes. The cells were pelleted by centrifugation at 400 x *g* for 5 min at room temperature, after which the supernatant was decanted and cells were re-suspended in 300 µl antibiotic-free IPEC-1 medium. 10 µl of a working dilution of the FAM-peptide-FMK caspase inhibitor was added and the tube was flicked to sufficiently mix the contents. The cells were incubated with the fluorescent inhibitor for 1 h at 37°C, 5% CO_2, protected from any source of light. After this time the cells were washed by adding 2 ml of the kit wash buffer and centrifuging at 400 x *g* for 5 min. The supernatant was decanted and the cell pellet was re-suspended in 400 µl wash buffer. To distinguish between live and dead cells, regardless of caspase activation, 5 µl propidium iodide (PI) was added to the cell suspension and incubated on ice for 5-10 min before immediate analysis by flow cytometry.

4.2.5.3 Analysis by flow cytometry

Unfixed, stained cell suspensions were analysed using a BD Accuri C6 flow cytometer (BD Biosciences, UK). Fluorescence gates were set using a sample of cells stained with FAM-peptide-FMK only, a sample stained with PI only and an unstained sample. All samples were analysed using the same fluorescence gate settings and the IPEC-1 cells of interest were expressed as a percentage of the total population. The FSC-H threshold was set to 500,000 and 10,000 events were recorded per sample.

4.2.6 Statistical analysis

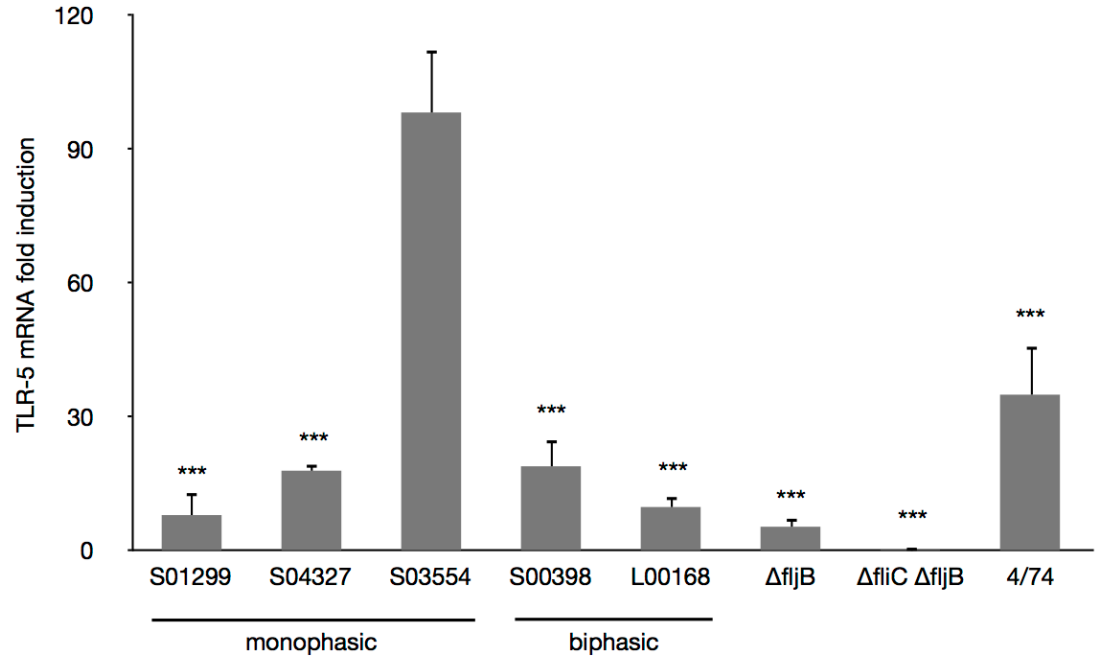
Statistical analyses were performed using MiniTab software, version 16. Mean values and standard deviations were calculated and isolates were compared using one-way ANOVA, unless stated otherwise. Differences were considered significant when $P<0.05$. In the event that a significant difference was found, the Tukey method of post-hoc multiple comparisons was performed.

4.3 Results

4.3.1 Expression of TLR-5 and IL-8 mRNA

The induction of a pro-inflammatory response from intestinal epithelial cells via flagellin-dependent stimulation of TLR-5 is a well-characterised signalling pathway (Elewaut *et al.*, 1999; Gewirtz *et al.*, 2001a; Gewirtz *et al.*, 2001b; Hayashi *et al.*, 2001; Tallant *et al.*, 2004; Yu *et al.*, 2003; Zeng *et al.*, 2003). Quantitative real-time PCR was used to determine whether monophasic expression of flagella affects host cell expression of TLR-5, the receptor for flagellin, and resultant induction of the pro-inflammatory chemokine IL-8. As shown in Figure 4.1A, upregulation of TLR-5 mRNA was detected in IPEC-1 cells 1 h post-infection in response to all *Salmonella* isolates, regardless of whether they were monophasic or not, with the exception of the aflagellate mutant (GC003, Δ*fliC* Δ*fljB*). In most instances, the increase in gene expression was modest (< 50-fold), but infection with 4,5,12:i:- isolate S03554 resulted in a significantly greater increase in TLR-5 expression (100-fold; P = 0.0000). IL-8 mRNA levels in IPEC-1 cells also increased in response to infection with all isolates studied here (Figure 4.1B).

A.


TLR-5 mRNA fold induction
120
90
60
30
0
***
S01299
S04327
S03554
S00398
L00168
ΔfljB
ΔfliC ΔfljB
4/74
monophasic
biphasic

B.

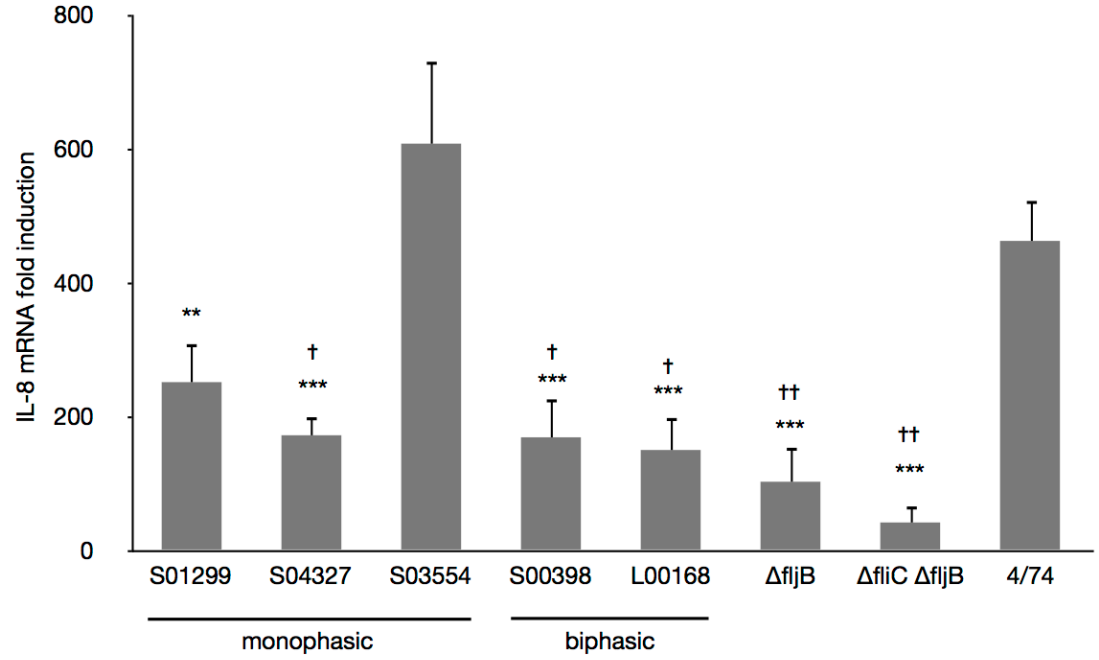

IL-8 mRNA fold induction
800
600
400
200
0
**
†
***
††
S01299
S04327
S03554
S00398
L00168
ΔfljB
ΔfliC ΔfljB
4/74
monophasic
biphasic

Figure 4.1 Comparison of induction of TLR-5 and IL-8 mRNA in *Salmonella*-challenged IPEC-1 cells.

IPEC-1 cells grown in tissue culture plates were infected for 1 h with *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and 4/74. RNA from challenge and control IPEC-1 cells was isolated and relative levels of TLR-5 (**A**) and IL-8 (**B**) mRNA were determined by quantitative real-time PCR. Data are means ± SEM of two independent experiments assayed in triplicate. PCR reactions were also performed in triplicate. Statistically significant differences from S03554 (*) and 4/74 (†) are indicated as follows: **, $P < 0.01$; ***, $P < 0.001$; †, $P < 0.05$; †† $P < 0.01$.

4.3.2 Activation of caspases

Previous studies have shown that flagellin-induced pro-inflammation is able to suppress apoptosis via activation of anti-apoptotic genes (Vijay-Kumar *et al.*, 2006; Zeng *et al.*, 2006). Given that the 4,[5],12:i:- isolates in this study were not attenuated in their ability to stimulate IL-8 mRNA expression it was hypothesised that similar levels of apoptotic activation would be seen in IPEC-1 cells challenged with monophasic and biphasic isolates. Analysis of cells treated with a fluorescent substrate (FAM-VAD-FMK) that binds to enzymatically active caspases (caspase-1, -2, -3, -4, -5, -6, -7, -8 and -9) by flow cytometry enabled differentiation between host cells with active apoptotic pathways and those without. Staining with PI, a membrane impermeant compound enabled further discrimination between viable and non-viable cells. Figure 4.2 is a representative flow cytometry data acquisition output showing fluorescence events detected in the FL1 (FAM-VAD-FMK) and FL3 (PI) channel.

At 2 h post-infection, levels of activated caspases (as determined by intensity of the fluorescent substrate) were increased in infected IPEC-1 cells compared to uninfected controls, although this did not reach statistical significance (Figure 4.3A and B). At this time point, 35-50% of IPEC-1 cells in each sample were positive for active caspases but no significant differences between the DT193 isolates were found (Figure 4.3C). The uninfected control cell populations had a similar proportion of caspase-positive cells to the challenged samples. The proportion of live cells positive for active caspases increased only marginally from 35-50% at 2 h to 48-58% at 8 hours (Figure 4.3C). Again, no differences were observed between the isolates at the later time point.

More cell death was observed in *Salmonella*-infected IPEC-1 cells compared to uninfected controls after 2 h (Figure 4.3D). No differences in cell death were observed between cells challenged with any of the DT193 isolates. The proportion of dead cells with detectable active caspases increased only slightly from 23-37% at 2 h to 29-40% at 8 h.

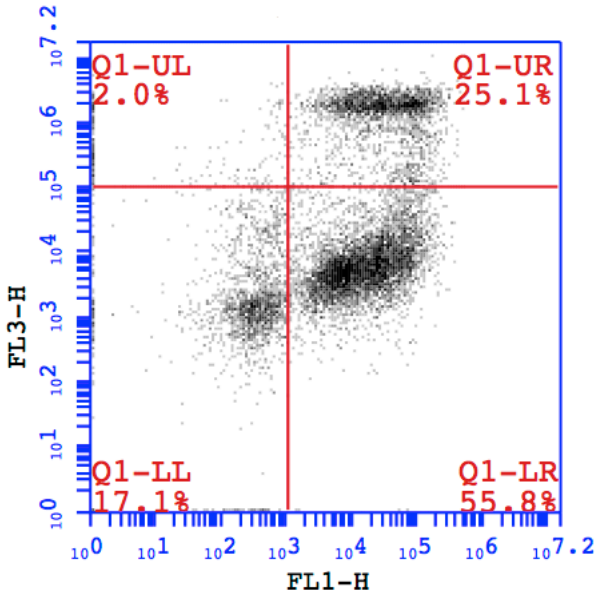
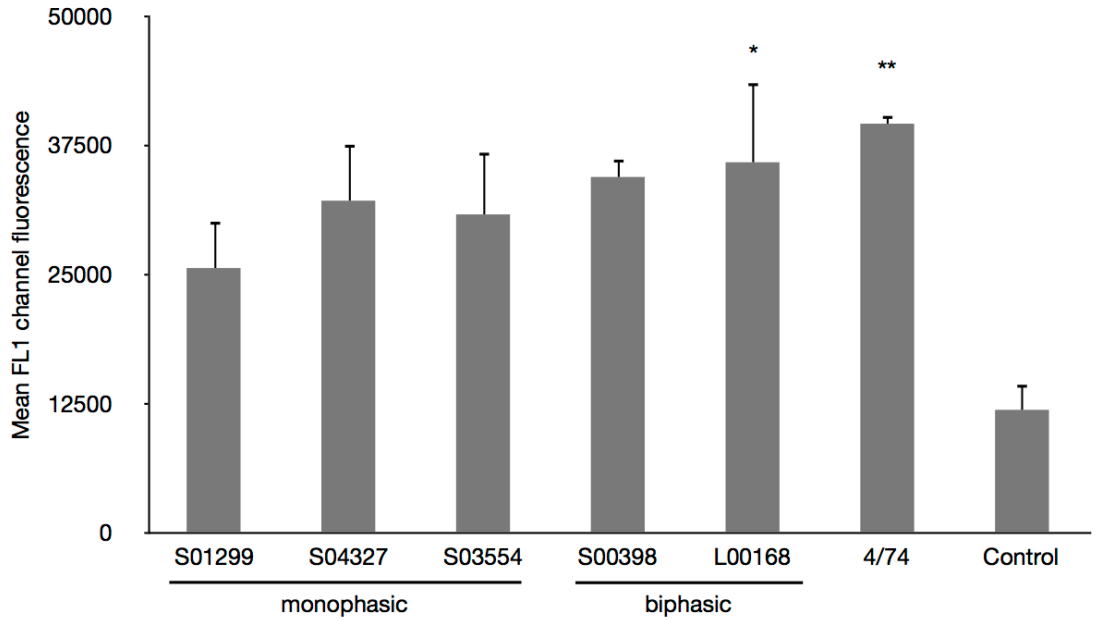


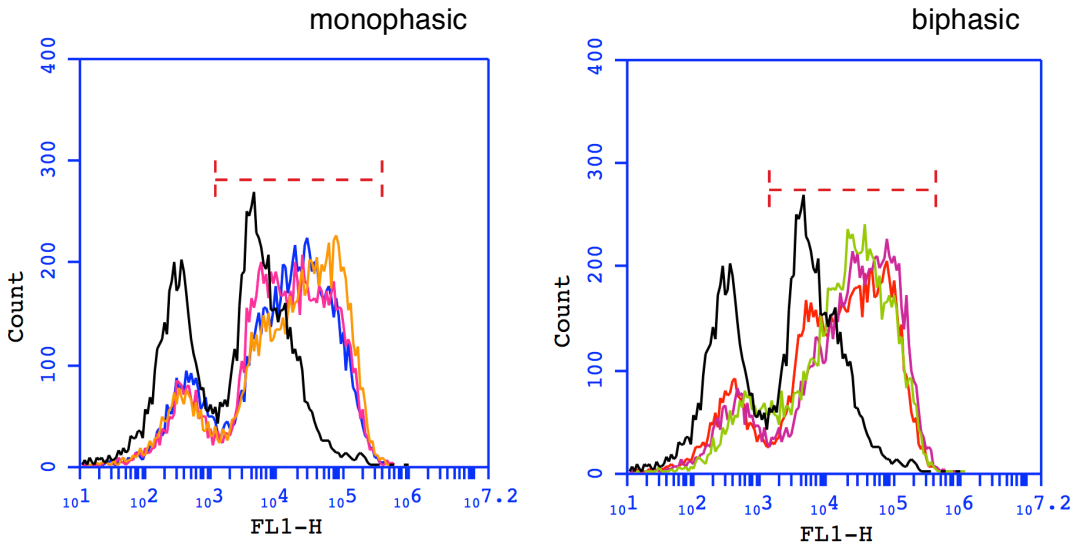
Figure 4.2 Proportion of *Salmonella*-challenged IPEC-1 cells with active caspases.

IPEC-1 cell monolayers grown in 24-well tissue culture plates were challenged with *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and 4/74. Harvested cell samples were fluorescently labelled with a substrate that binds to caspases -1 to -9 (FAM-VAD-FMK). Cells were also stained with propidium iodide (PI) to differentiate between live and dead cells. Representative flow cytometer (BD Accuri C6) data acquisition output showing fluorescence events detected in the FL1 channel (FAM-VAD-FMK) and FL3 channel (PI). Lower-right quadrant contains live, caspase-positive cells; upper-right quadrant contains dead, caspase-positive cells.

A.

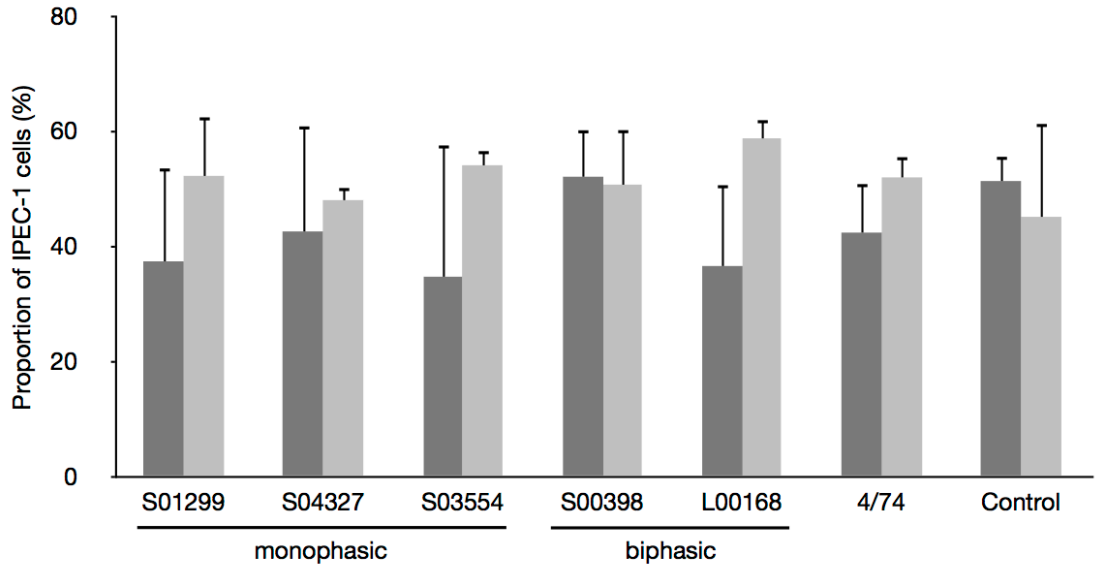

Mean FL1 channel fluorescence
50000
37500
25000
12500
0
*
**
S01299
S04327
S03554
S00398
L00168
4/74
Control
monophasic
biphasic

B.


monophasic
biphasic
Count
400
300
200
100
0
10^1
10^2
10^3
10^4
10^5
10^6
$10^{7.2}$
FL1-H

C.

live, caspase-positive cells



D.

dead, caspase-positive cells

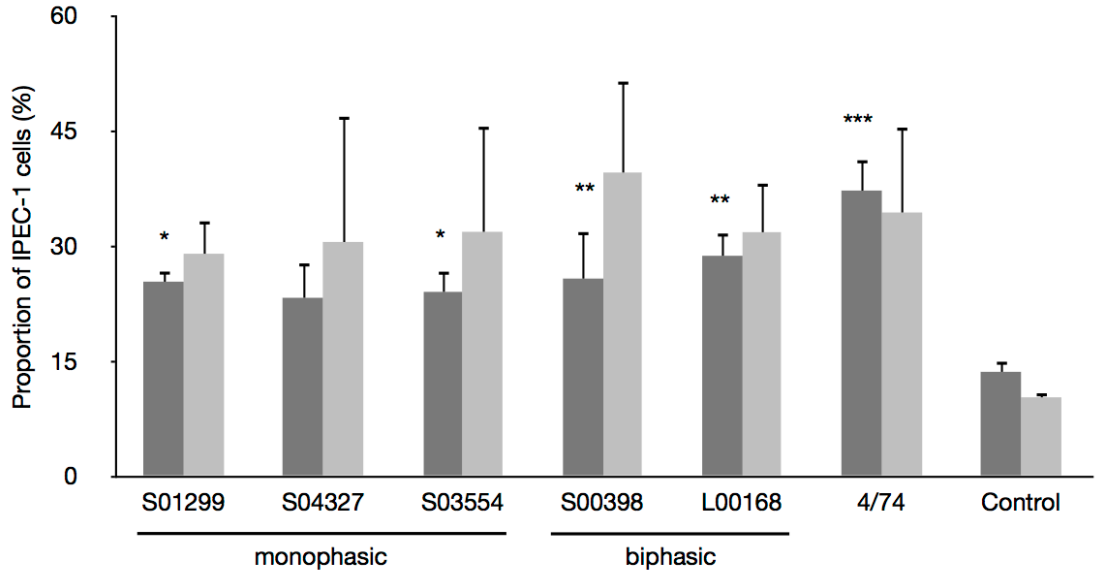


Figure 4.3 Levels of detectable active caspases in *Salmonella*-challenged IPEC-1 cells.

IPEC-1 cell monolayers grown in 24-well tissue culture plates were challenged with *Salmonella* 4,[5],12:i:- and Typhimurium DT193 isolates and 4/74. Cells were harvested 2 h (■) or 8 h (■) post-infection and active caspases were labelled with a fluorescent inhibitor (FAM-VAD-FMK). **A**. Mean fluorescence intensity of FAM-VAD-FMK detected in the FL1 channel. **B**. Representative flow cytometry data acquisition outputs showing fluorescence intensity (x-axis) and the number of detection events (y-axis) in samples of cells infected with monophasic (left) and biphasic (right) isolates 2 h post-infection. The coloured lines represent outputs from infected samples and the black line represents output from uninfected control cells. The red dashed marker indicates the caspase (FAM-VAD-FMK)-positive proportion of the population. **C**. Proportion of live IPEC-1 cells with detectable active caspases (FAM+PI-). **D**. Proportion of dead IPEC-1 cells with active caspases (FAM+PI+). Samples were analysed using a BD Accuri C6 flow cytometer, with 10,000 fluorescent events measured per sample. Results are mean ± SD of three independent experiments. Statistically significant differences from uninfected control cells are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P = 0.0000$.

4.4 Discussion

This study investigated the ability of monophasic and biphasic *Salmonella* DT193 isolates to stimulate specific arms of the innate immune response associated with intestinal epithelial cells in a porcine *in vitro* model: upregulation of TLR-5 and IL-8 mRNA and caspase activation. Tissues of the porcine intestinal tract have been shown to upregulate expression of both TLR-5 and IL-8 in response to *S.* Typhimurium infection *in vivo* (Burkey *et al.*, 2007; Cho & Chae, 2003; Skjolaas *et al.*, 2006; Uthe *et al.*, 2007; Vo *et al.*, 2007) and *in vitro* (Skjolaas *et al.*, 2006; Volf *et al.*, 2007).

Consistent with Zeng *et al.* (2003), who found that monophasic mutants of *S.* Typhimurium were able to stimulate full pro-inflammatory gene expression profiles from human colonic epithelial cells, results presented in this chapter show that monophasic *Salmonella* DT193 induce an increase in TLR-5 and IL-8 mRNA expression levels in porcine intestinal epithelial cells. No differences in TLR-5 or IL-8 expression were found between monophasic and phase variable DT193 isolates, except S03554, which elicited significantly higher levels of both. However, in another study, genetically engineered *S.* Typhimurium strains lacking second phase flagella were shown to have reduced ability to stimulate an innate immune response from cultured human intestinal epithelial cells compared to the wild-type strain (Gewirtz *et al.*, 2001). This is in conflict with the results presented here, although the difference in model host species could be a factor.

To confirm that the TLR-5 and IL-8 results were not exclusive to the particular 4,[5],12:i:- isolates chosen for this study, IPEC-1 cells were also challenged with GC002, an isogenic monophasic mutant (Δ*fljB*) of L00168 created by P22 transduction (Chapter 2, Section 2.2.5). As described in Chapter 2 (Section 2.3.3), examination of GC002 by TEM revealed that its flagella are stunted in length compared to the parent strain. This isolate also has greatly reduced motility compared to the parent strain (Chapter 2, Figure 2.3E). A possible explanation is that although the *fljB* gene was knocked-out of the parent strain the *fliC* repressor gene, *fljA*, could still be expressed, resulting in reduced expression of FliC flagella protein (Aldridge *et al.*, 2006). However, despite its reduced motility, infection with the Δ*fljB* mutant elicited upregulation of both TLR-5 and IL-8 mRNA (~5-fold and ~100-fold, respectively). The upregulation was to a lesser degree than that caused

by the parent strain (L00168) but the difference was not significant (P = 0.9998 and P = 0.9994). In line with this result, it has been shown elsewhere that motility is not essential for stimulation of a pro-inflammatory response (Zeng *et al.*, 2003). The limited upregulation could be due to a reduced amount of FliC flagellin protein being present. Overall, results obtained following infection with the Δ*fljB* mutant support the theory that *Salmonella* exhibiting monophasic expression of FliC can stimulate TLR-5 and IL-8 upregulation as potently as biphasic DT193 isolates.

Flagellin protein has a four-domain structure; D0 and D1 are highly conserved domains located at the N and C termini of the primary sequence, whereas the D2 and D3 domains comprise a central hypervariable region. It has previously been shown that only the conserved domain of flagellin is required for TLR-5 activation (Eaves-Pyles *et al.*, 2001; Smith *et al.*, 2003; Yoon *et al.*, 2012), suggesting that the receptor would be unable to discriminate between FliC and FljB. Similarly, purified FliC and FljB have been reported to activate similar levels of NF-κB, a transcription factor activated by TLR-5, in cultured cells (Simon & Samuel, 2007). The ability of the 4,[5],12:i:- isolates in this study to stimulate TLR-5 expression, despite lacking expression of FljB, is in keeping with these findings.

The aflagellate mutant stimulated a 40-fold increase in IL-8 expression, despite a lack of TLR-5 induction. A previous study found that an aflagellate mutant of *S.* Typhimurium over-expressing the SPI-1 T3SS-1 effector protein, SopE2 was able to induce IL-8 from T84 cells (Huang *et al.*, 2004), suggesting that some activation of IL-8 is possible in the absence of flagellin. Indeed, recognition of other PAMPs, such as CpG-DNA, may also induce IL-8 production (Ewaschuk *et al.*, 2007).

The exceptionally high TLR-5 mRNA levels observed following infection with S03554 correlated with similarly high induction of IL-8 expression (~600-fold increase). It is possible that changes in TLR-5 and IL-8 expression during infection with this particular isolate follow a different time course to those during infection with the other isolates; analysis of gene expression at only one time point in this study limits the ability to investigate this theory. Alternatively, the increased IL-8 expression seen with S03554 may be a result of this strain's specific hypervariable domain sequence. Indeed, Liu *et al.* (2010a) reported that a FliC flagellin protein variant with a deletion in the hypervariable domain elicited higher production of IL-8 and TNF-α from Caco-2 cells than full length FliC. However, it has also been shown that the

hypervariable domain is not mandatory for TLR-5 signalling (Eaves-Pyles *et al.*, 2001; Smith *et al.*, 2003) so the heightened TLR-5 expression cannot be explained in this way. Given that the majority of isolations of *Salmonella* from pigs in the UK result from the investigation of clinically diseased animals and the number of isolations of 4,[5],12:i:- strains from pigs is increasing (Anon., 2013a), it is thought that monophasic *Salmonella* are capable of causing clinical symptoms in these animals (R. H. Davies, personal communication). Usually, *Salmonella* infection is asymptomatic in pigs and the possibility that monophasic strains cause clinical disease is an emerging pig health concern. Indeed, the particularly strong induction of IL-8 by S03554 in this study could result in a greater than normal influx of neutrophils to the site of infection, which would potentially cause considerable intestinal inflammation and diarrhoea. However, investigation of this during *in vivo* infection is required to substantiate this theory.

The pro-inflammatory response of epithelial cells to invading pathogens is only part of the host innate immune response. Activation of caspases, the initiators of programmed cell death, is another response of epithelial cells to flagellin-derived TLR-5 stimulation and occurs in parallel to pro-inflammation (Zeng *et al.*, 2006). If this arm of the response was permitted to proceed without regulation, infected cells would undergo apoptosis, presumably as a mechanism of eradicating bacteria from the host. However, unimpeded apoptosis of intestinal epithelial cells would ultimately result in severe tissue damage, so recognition of flagellin also activates anti-apoptotic processes in epithelial cells in order to delay apoptosis (Vijay-Kumar *et al.*, 2006). The proportion of dead IPEC-1 cells increased only marginally between 2 h and 8 h, suggesting that despite a large proportion of the total cell population having detectable active caspases, the rate of programmed cell death was low, perhaps due to the aforementioned regulation of apoptosis. This is consistent with the findings of others who found that anti-apoptotic processes are also activated in epithelial cells during *Salmonella* infection as a mechanism of delaying programmed cell death (Liu *et al.*, 2010b; Vijay-Kumar *et al.*, 2006; Zeng *et al.*, 2006).

The activation of caspases observed here seems to have been more rapid than in other studies. For example, Vijay-Kumar *et al.* (2006) observed only very low activation of caspase-3, -8 and -9 after 2 h infection. However, it is possible that the early caspase activation seen in this study is a result of using a fluorescent peptide that binds to caspases -1 to -9 for detection. The non-specific nature of the

fluorescent reagent may also explain why the proportion of live cells with active caspases increased only marginally between 2 h and 8 h. Since the fluorescent substrate used binds to all active caspases, it was not possible to determine which specific caspases were responsible for the fluorescent signal. Indeed, some caspase activation might have occurred as part of inflammatory pyroptosis, which involves caspase-1, or independently of infection as part of routine cellular development and proliferation within the cultured monolayer (Schwerk & Schulze-Osthoff, 2003). Epithelial cells infected with *S*. Typhimurium have been reported to activate caspase-1 (Knodler *et al.*, 2010), the primary initiator of an alternative programme of cell death known as pyroptosis, which is usually associated with *Salmonella*-infected macrophages (reviewed in Fink & Cookson, 2007). Further investigation is needed to determine whether the relatively high proportion of cells with active caspases at 2 h post-infection observed in this study was due to faster activation, or activation of a wider repertoire of caspases. What is clear from the results, however, is that there is no difference between monophasic and biphasic DT193 isolates in their ability to stimulate caspase activation or in their cytotoxicity. Therefore, lack of FljB expression by 4,[5],12:i:- isolates may not have a substantial effect on the delicate equilibrium between pro-inflammation and apoptosis.

Taken together, the data presented in this chapter demonstrate that isolates of *Salmonella* 4,[5],12:i:- and Typhimurium belonging to phage type DT193 and originating from pigs are capable of stimulating aspects of the typical innate immune response from porcine intestinal epithelial cells. Upregulation of genes involved in pro-inflammation was detected in epithelial cells challenged with these isolates, as was activation of caspases. Except for one 4,5,12:i:- isolate that elicited significantly greater TLR-5 and IL-8 upregulation, no differences between monophasic and biphasic isolates in stimulation of pro-inflammation or activation of apoptosis were detected, suggesting that monophasic expression of FliC may not significantly affect either of these immune response pathways in porcine intestinal epithelial cells.

5 General Discussion

The main findings presented in this thesis are as follows. The panel of DT193 isolates possessed a heterogeneous repertoire of virulence-related phenotypes and genotypes. The 4,[5],12:i:- isolates exhibited comparable adhesion and invasion to that of the virulent *S*. Typhimurium isolate 4/74, suggesting that these strains may be capable of colonising the small intestine of pigs *in vivo*. Infection with 4,[5],12:i:- and biphasic DT193 isolates resulted in approximately the same level of TLR-5 and IL-8 mRNA upregulation, except in the case of one 4,5,12:i:- isolate that elicited significantly greater upregulation of these genes. The monophasic variants also elicited similar levels of caspase activation and cytotoxicity to the phase variable DT193 isolates. These results suggest that monophasic *Salmonella* display a similar infection biology to phase variable *S*. Typhimurium during colonisation of the porcine intestinal tract. Consequently, it seems that flagellar phase variation may not be important during the early intestinal stage of infection in pigs.

Salmonella Typhimurium phage type DT193 and monophasic *Salmonella* belonging to the same phage type are increasing in prevalence across Europe. They are now among the most commonly isolated serotypes of *Salmonella* from both pigs and humans (Anon., 2013c; Anon., 2014a) and they display wide resistance to antimicrobials. The monophasic variants, designated serotype 4,[5],12:i:-, are characterised by their lack of expression of the second phase flagellar antigen. Although the biological significance of flagellar phase variation, exhibited by *S*. Typhimurium and other serotypes, is not understood, it is thought that this phenotype confers virulence (Aldridge *et al*., 2006; Ciacci-Woolwine *et al*., 1998; Ikeda *et al*., 2001; Yamamoto & Kutsukake, 2006). Therefore, the ecological success of monophasic *Salmonella* isolates is puzzling and, indeed, very little is known about the pathogenicity of these strains. A primary aim of this thesis was to compare genotypic and phenotypic features of biphasic *S.* Typhimurium and 4,[5],12:i:- DT193 isolates. Because the main reservoir species of 4,[5],12:i: strains appears to be pigs (Hopkins *et al*., 2010) and because pigs and pork represent a significant source of human salmonellosis (Pires *et al*., 2010; Pires *et al*., 2011), isolates collected from pig faeces on British pig farms were analysed. This work also aimed to compare monophasic and biphasic strains in their ability to adhere to and invade porcine intestinal epithelial cells and to characterise the immune response of the host cells to the invading bacteria.

While a substantial number of studies have characterised vast numbers of the 4,[5],12:i:- isolates emerging worldwide by molecular techniques, few have characterised their phenotype in such a way as is presented in this thesis. It was found that the DT193 isolates studied here do not share a distinct phenotype or genotype. Instead, like *S.* Enteritidis isolates which display great phenotypic diversity even within the same phage types and clonal lineages (Clavijo *et al.*, 2006; Shah *et al.*, 2011; Yim *et al.*, 2010), different virulence profiles were identified. For instance, the results of the virulotyping revealed heterogeneous possession of *sopB*, *sopE* and *pefA* among the DT193 isolates. These differences did not translate into easily identifiable differences in adhesion, invasion or stimulation of immune response. Another interesting phenotypic difference was in the biofilm-forming abilities of the isolates, where certain strains seemed more adapted to rapid biofilm formation at 37°C and others to slow biofilm formation at ambient temperature.

Before this study the infection biology of monophasic, and indeed biphasic, DT193 isolates in a porcine model had not been investigated. Genetically engineered monophasic mutants and phase-locked derivatives of *Salmonella* have been studied in models of other host animals, but the pathogenicity of naturally-occurring monophasic isolates has only been studied in the chicken (Martelli *et al.*, 2014; Parsons *et al.*, 2013). The results presented in this thesis demonstrate that both 4,[5],12:i:- and Typhimurium DT193 isolates are capable of adhering to and invading porcine intestinal epithelial cells. The monophasic isolates showed no evidence of an enhanced capacity for adhesion and invasion of pig or human epithelial cells, or of murine macrophage-like cells compared to the biphasic strains. Additionally, no differences in stimulation of pro-inflammatory gene expression between the monophasic and biphasic isolates were found, except in the case of one monophasic isolate that elicited significantly greater upregulation of TLR-5 and IL-8 mRNA. The ability of the 4,[5],12:i:- isolates to stimulate TLR-5 and IL-8 expression, despite their lack of *fljB* expression is in keeping with the findings of others (Simon & Samuel, 2007; Zeng *et al.*, 2003). Finally, no differences between monophasic and biphasic isolates in their stimulation of caspase activation or cytotoxicity were found. Together, these results suggest that flagellar phase variation may not be important during the early intestinal stage of infection in pigs, which is in agreement with conclusions from similar experiments investigating phase-locked *Salmonella* mutants in murine and bovine models (Ikeda *et al.*, 2001).

It is important to gain an understanding of the phenotype of emerging pathogens to gain clues as to how they are transmitted and how they might be controlled. For example, the ability of some of the isolates in this study to form biofilms at ambient temperatures may explain their widespread dissemination. Indeed, in a recent slaughter pig survey, over 50% of carcass swab samples were found to be monophasic *Salmonella*, suggesting that these strains are adapted to survive in a slaughterhouse environment (Anon., 2014b); persistence within biofilms on abattoir surfaces may be one such adaptation. Knowledge such as this could inform cleaning and disinfection protocols in pork processing plants in order to limit cross-contamination of pig carcasses with *Salmonella* and ultimately limit the prevalence of this bacteria on pork at retail. In the farm environment, enhanced survival of monophasic and biphasic *Salmonella* DT193 isolates within pig faeces, which has been shown to occur by Rajtak *et al*. (2012), coupled with survival within biofilms on the walls and floors of pig pens would likely contribute to spread throughout herds. Following confirmation of these results, farmers could be advised on improved slurry handling and cleaning practices to ensure the cycle of transmission of *Salmonella* DT193 isolates is broken. Further work to elucidate more about the environmental survival of these isolates, such as biofilm formation on stainless steel and the efficacy of disinfectants at a range of temperatures against them, would be beneficial. In addition, it would be interesting to sample key risk points for *Salmonella* contamination in pig abattoirs, such as dehairing equipment and meat knives, and serotype any positive *Salmonella* isolates to determine whether certain strains have an enhanced capacity for survival in the meat processing environment.

An understanding of host-pathogen interactions in infected animals is also important for informing control measures. While immunological control of host-restricted serotypes of *Salmonella* using vaccines has proven successful to date, similar vaccines have been less effective in protecting against broad-host range serotypes in food-producing animals. Barrow (2007) hypothesises that this may be due to the differences in pathogenesis exhibited by the two groups of *Salmonella*. Host-restricted serotypes cause systemic disease with poor intestinal colonisation and therefore cell-mediated immunity plays a significant role in clearance of the bacteria from host tissues. Whereas, infection with broad host-range serotypes involves considerable intestinal colonisation resulting in a more complex host-pathogen interaction and immune response. Colonisation of the intestinal tract during

Salmonella infection is a multifaceted process, relying on a large number of different genes encoding secreted and surface-anchored molecules, some of which are more or less important depending on the host species (Carnell *et al.*, 2007). Because immune responses to *Salmonella* differ depending on the serotype and the host species involved, it is important to characterise intestinal colonisation in all relevant host and serotype models and to express caution when extrapolating results from one model to another (Barrow, 2007). That both the monophasic and biphasic DT193 isolates were able to attach and invade porcine intestinal epithelial cells and stimulate a pro-inflammatory response is characteristic of traditional *S*. Typhimurium infection in pigs and suggests that vaccination against this phage type would offer only minimal protection. However, development of vaccines to reduce faecal shedding of this strain and consequentially limit transmission and contamination may be possible.

S. Enteritidis infection in chickens involves colonisation of the reproductive tract and internal egg contamination (De Buck *et al.*, 2004), which has caused large numbers of egg-related infections in humans historically. *Salmonella* infection in laying hens has now been successfully controlled in the UK due to implementation of a NCP involving hygienic measures and the testing and removal of positive flocks from production. This has resulted in a dramatic decline in the number of cases of *S*. Enteritidis infection in humans (Cogan & Humphrey, 2003; Griffin & O'Brien, 2013). There are a number of vaccines, live and killed, available commercially for use in laying hens against *S.* Enteritidis and *S.* Typhimurium (Clifton-Hadley *et al.*, 2002; Gantois *et al.*, 2006; Springer *et al.*, 2011), however vaccination in this setting is regarded only as an additional measure to increase resistance to exposure and reduce egg contamination (Anon., 2004). In a recent study, vaccination was shown to be effective at reducing egg contamination in laying hens experimentally challenged with tetra-resistant 4,[5],12:i:- isolates, but it had no effect on the proportion of birds shedding *Salmonella* compared to non-vaccinated birds (Arnold *et al.*, 2014). This is noteworthy because the same pig-derived monophasic DT193 isolates used in this study have been shown to colonise the caeca and liver of chickens (Parsons *et al.*, 2013). Monophasic *Salmonella* has been isolated from a small number of chicken breeding and laying flocks in the UK, all of which had pigs on the same or adjacent holding (Davies, 2011). The spread of monophasic *Salmonella* from pigs to chickens is a significant and emerging threat to poultry

production, particularly since May 2011 when legislation surrounding the laying hen NCP (EC 517/2011) was updated such that isolation of 4,[5],12:i:- from a flock triggers the same restrictions as the isolation of *S*. Enteritidis and *S.* Typhimurium.

While development of a vaccine to protect against monophasic and biphasic DT193 isolates may be useful, in the time it takes for one to reach the market the threat of these strains may no longer be apparent, if the epidemiology of epidemic *S.* Typhimurium strain DT104 is anything to go by. *Salmonella* DT193 isolates display a number of similar characteristics to DT104, all of which probably contribute to their epidemic potential. For example, both phage types harbour chromosomally encoded antibiotic resistance genes and both have demonstrated widespread dissemination. Since its emergence in the 1990s and peak in prevalence in 1997, there has been a steady decline in the frequency with which DT104 is isolated (Miller *et al.*, 2011). The exact reason for this decline is not known, however it is possible that following its dissemination the livestock populations in which it circulated developed natural immunity, thus limiting further infections. There is a possibility that European pig herds may eventually become resistant to DT193 and this phage type may experience a similar decline in prevalence.

In the meantime, it would be pertinent to further investigate the infection biology of both monophasic and biphasic DT193 to understand more about their ecological success and to inform control strategies, especially considering their resistance to multiple antibiotics and their frequent isolation in humans. Indeed, treatment of human salmonellosis may become increasingly difficult as these strains acquire further antimicrobial drug resistance by horizontal transfer of genes. The conclusion in this thesis that flagellar phase variation does not contribute significantly towards *Salmonella* pathogenicity during the early stages of intestinal colonisation opens up further questions to be answered. For example, are the results from this study a consequence of the use of a cell model that is only a simplistic representation of the pig intestine? Collado-Romero and colleagues (2010) identified that there is high variation along the porcine gut in the immune response to *S*. Typhimurium infection, with greater expression of certain pro-inflammatory cytokine transcripts in the jejunum and colon than in the ileum. While the IPEC-1 cell line used in this study is a mixture of ileal and jejunal cells, it would be interesting to characterise the immune response of the different regions of the porcine gut following *in vivo* infection. It may

be that monophasic expression of flagellar antigen confers a selective advantage during infection of some tissues, but not others.

Analysis of the innate immune response by q-PCR or whole genome microarray could be combined with immunohistochemistry of tissue samples to characterise the humoral response. It would be particularly interesting to further explore the pathogenicity of isolate S03554 and the host response to this inflammatory strain, perhaps in *in vivo* animal infection experiments. Quantification of the immune response at additional time points would also be valuable, to determine host-pathogen interactions as the infection progresses. Investigation of adhesion, invasion and persistence of monophasic strains in porcine tonsillar tissue would be another interesting infection site to explore, given that the tonsils have been implicated in maintenance of the subclinical carrier state of infection in pigs (Wood et al., 1989). It is interesting to note that both *fliC* and *fljB* were upregulated by *S.* Typhimurium when persisting in porcine tonsils, but not in the ileum or ileocaecal lymph nodes, during *in vivo* infection in pigs (van Parys et al., 2011). The role of these flagellar proteins and phase variation during different stages of *Salmonella* infection and during interaction with different tissues and hosts is clearly complex. It is therefore important to elucidate their involvement in all relevant experimental settings. Comparison of the infection biology of monophasic and biphasic strains beyond the intestinal epithelium would also enable determination of their ability to cause systemic disease and the role of flagellar phase variation in this stage of infection. It could be that monophasic expression of FliC confers a selective advantage enabling systemic spread and/or maintenance of a carrier state. Primary culture of porcine monocytes and investigation into the invasion, persistence and replication of the *Salmonella* isolates in these cells would provide a host-specific extension to the work with murine macrophages presented in this thesis.

While this study gave some insight into the distribution of *Salmonella* bacteria within a monolayer of intestinal epithelial cells, with some evidence of targeted invasion being apparent, further work on this could be done. The proliferation, spread and distribution of *Salmonella* in different organs has been studied during *in vivo* infection of mice with the aid of mathematical models (Mastroeni *et al.*, 2009). Investigation of the spatiotemporal pattern of spread of DT193 isolates and sites of bacterial persistence within the porcine host may provide further understanding of the pathogenicity of these strains and enable efficient targeting of bacteria with

antimicrobials, bacteriophage or vaccine-induced immune responses. Detailed analysis of the host cells that experience hyperinvasion of bacteria would be useful to help understand this phenomenon. For example, actin staining of cells coupled with fluorescent microscopy would enable investigation of cellular morphology and the involvement of membrane ruffles.

An alternative approach to that taken in this study, whereby different aspects of *Salmonella* virulence were investigated in a suppositional manner, would be to first identify discrepancies between host tissue responses to monophasic and biphasic DT193 isolates and then focus on further elucidating those. This could be done by comparing the transcriptome of the intestinal epithelium in response to monophasic isolates to the transcriptome in response to biphasic isolates; any differences would point to specific pathways and mechanisms for further investigation. Similarly, it would be worthwhile performing RNA-seq-based transcriptomic analysis of the isolates themselves in different environments related to invasion of the host. Previous application of this approach has shown elegantly that *Salmonella* Typhimurium gene expression is very sensitive to environmental conditions and it is possible to characterise the different expression patterns in a variety of contexts (Kröger *et al.*, 2013). It would also be interesting to sequence the complete genomes of a number of monophasic and biphasic DT193 isolates and make comparisons between them and with other phage types of *S*. Typhimurium that are less prevalent. By this method it may be possible to identify lineage-specific genetic factors that account for lineage-specific phenotypes. For example, high-throughput genome sequencing would facilitate analysis of single-nucleotide polymorphisms (SNPs) and their significance in terms of bacterial virulence. Alignment and analysis of the genome sequences may draw attention to, for example, the presence of virulence-conferring phage. Analysis of large numbers of *S*. Typhimurium DT193 isolates by high-throughput genomic, transcriptomic and proteomic methods would significantly extend the work in this thesis, which is limited by the small sample of isolates and their individual variations.

In conclusion, monophasic and biphasic isolates of *Salmonella* phage type DT193 present in British pig herds present a considerable threat to both the pig and poultry industry and to public health. These strains are capable of invading porcine intestinal epithelial cells and eliciting aspects of the typical innate immune response from these cells. Very few differences between monophasic and phase variable

strains were identified and so further work is needed to understand the selective pressure behind the loss of the second flagellar antigen by 4,[5],12:i:- isolates circulating Europe.

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Appendix 1

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